## REMARKS

Reconsideration of this application is requested in view of the amendments to the claims and the remarks presented herein.

The claims in the application are claims 29, 30, 32, 38 to 40, 42 to 44 and 47, all other claims having been cancelled.

Claims 29 to 32, 38 to 40, 42 to 44 and 47 were rejected under 35 USC 112, first paragraph, as containing subject matter which was enabled by the specification. The Examiner objects to page 7 as not defining a variable m or n and picking and choosing the variable numbers than can be encompassed thereby. The Examiner is of the opinion that the specification fails to provide a written description for a vaccine or immunogenic composition effective against tumors and that the specification fails to provide an adequate description of the different derivatives of the carbohydrate tumor antigen. The Examiner is further of the opinion that the claims do not recite a specific tumor and there is nothing to extrapolate the illustrated tumors of Longenecker as Applicants have argued. The Examiner incorrectly states that Applicants concede that the Examiner is correct which she underlines that the claimed carbohydrate peptide conjugates will not be effective for treating all types of cancer. The Examiner is of the opinion that the claimed carbohydrate peptide conjugates consist of a combination of the specific structures of the

dendrimeric poly lysine residues with a lysine core combined with the presence of a B-cell epitope consisting of a carbohydrate moiety.

Applicants respectfully traverse these grounds of rejection since it is believed that the present claims are properly enabled by the specification. Claim 29 has been limited to a more specific carbohydrate peptide conjugate which induces an antibody response against a carbohydrate tumor antigen wherein the conjugate is selected from formulae (a) to (d) and wherein (b) is a carbohydrate moiety containing a tumor antigen or a carbohydrate derivative thereof, T is a $CD4^+$ T cell peptide epitope and K is a lysyl residue. It is deemed that claim 29 as presently presented strictly relates to a carbohydrate peptide conjugate which induces an antibody response against a carbohydrate tumor antigen and support for this can be found in many places in the specification. The Examiner's attention is directed to lines 26 to 28 of page 4, lines 17 to 19 of page 6 and Examples 2, 3 and 4. Therefore, it is deemed that the claims as presently presented are properly enabled. Withdrawal of this ground of rejection is requested.

Claims 29 to 32, 38 to 40, 42 to 4 and 47 were rejected under 35 USC 112, second paragraph, as being indefinite for reasons of record. The Examiner notes that B is indefinite since there is no structure defined in the claims for the carbohydrate tumor antigen. The Examiner makes reference to claim 35 which has been cancelled. Claim 40 is deemed indefinite for not reciting the other components of the vaccine since in the

absence of differentiating components, the composition is the same as the immunogenic compositions.

Applicants respectfully traverse these grounds of rejection since it is deemed that the amended claims properly comply with 35 USC 112, second paragraph. With respect to the expression "derivatives", the Examiner erroneously reports that it is "it is not apparent whether a derivative i.e., among a modified form of the carbohydrate would still affect the desired effect as noted on page 10 of the office action. Applicants deem that Example 1 of the application discloses a method for the synthesis of the Tn antigen by a completely chemical procedure. However, the completely synthetic Tn antigen is not the exact natural product that would have been extracted from the cell membrane of the tumor cell expressing the same has entirely preserved its antigenicity since it is recognized by antibodies specific to the natural Tn antigen as can be seen from Example 2. Moreover, this completely synthetic chemical Tn antigen is also immunogenic since it induces in vivo and effective antibody response that decreases the mortality of tumor bearing animals immunized therewith as can be seen from Example 3.

Any modification in the chemical synthesis of the Tn antigen is easily available to one skilled in the art as well as the possibility to check by routine procedures as disclosed in Examples 1 to 4 that the modified Tn antigen has the derivative of the Tn antigen preserves its antigenicity and immungenicity. The essential feature of the claimed peptide-carbohydrate conjugate does not consist in the structure of the tumor

carbohydrate moiety or the derivative thereof that is used but on the general structure of the conjugate which allows an effective antibody response at a level that was not reached with any of the immunogenic structures disclosed in the prior art.

With respect to the efficacy of the claimed peptide-carbohydrate conjugates for treating cancer, the same will be efficient in preventing, treating or at least reducing the incidence of a cancer in an animal body. Each time said cancer will be efficiently prevented or reduced to the raise of an antibody response specific to a carbohydrate antigen specifically expressed by the tumor cells. Applicants are submitting herewith six articles that clearly support the usefulness of the claimed peptide carbohydrate conjugates for treating cancer.

1) R. Lo-Man, S. Bay, S. Vichier-Guerre, E. Deriaud, D. Cantacuzene, C. Leclerc. A fully synthetic immunogen carrying a carcinoma-associated carbohydrate for active specific immunotherapy, Cancer Reasearch. 59, 1520-1524, 1999.

2) S. Vichier-Guerre, R. Lo-Man, S. Bay, E. Deriaud, H. Nakada, C. Leclerc, D. Cantacuzene. Short synthetic limear glycopeptides induce antibody responses to carcinoma associated Tn antigen, Journal of Peptide Research, 55, 173-180, 2000.

3) R. Lo-Man, S. Vichier-Guerre, S. Bay, E. Deriaud, D. Cantacuzene and C. Leclerc. Anti-tumor immunity by a synthetic multple glycopeptide displaying a Tri-Tn glycotpe.

4) S. Vichier-Guerre, R. Lo-Man, L. BenMohamed, E. Deriaud, S. Kovats, C. Leclerc, S. Bay. Induction of carbohydrate-specific antibodies in HLA-DR transgenic mice by a synthetic glycopeptide: a potential anti-cancer vaccine for human use. Journal of Peptide Research 62(3), 117-124, 2003.

5) S. Vichier-Guerre, R. Lo-Man, V. Huteau, E. Deriaud, C. Leclerc, S. Bay. Synthesis and immunological evaluation of an anti-tumor neoglycopeptide vaccine earing a novel homoserine Tn-antigen. Bioorganic and Medicinal Chemistry Letters, 14, 3567-3570, 2004.

6) R. Lo-Man, S. Vichier-Guerre, R. Perraut, E. Deriaud, V. Huteau, O.M. Diop, P. Livingston, S. Bay and C. Leclerc. A fully synthetic therapeutic vaccine candidate targeting carcinoma-associated Tn carbohydrate antigen induces tumor specific antibodies in non-human primates, Cancer Research, 64, 4987-4994, 2004.

Document No. 1 discloses the effective induction of anti-Tn antibodies by a peptide-carbohydrate conjugate of the invention as well as the usefulness for increasing mouse survival and resistance to tumor challenge as pointed out on page 1522 thereof.

Document No. 2 clearly shows the successful induction of an antibody response to carcinoma-associated Tn antigen with a peptide carbohydrate conjugate of the invention. Further anti-tumor activity of a peptide-carbohydrate conjugate of the invention are disclosed in Documents 4 to 6.

Document No. 6 shows the anti-tumor activities against the human tumor cell line of a modified Tn antigen, namely, a novel homocerine Tn antigen which consists of a derivative of the Tn antigen. Figure 2 clearly shows that a human tumor cell line is actually recognized by antibodies contained in the sera from mice prime to the peptide-carbohydrate conjugate containing said Tn antigen derivative. Moreover, Document No. 7 discloses results that prove a peptide-carbohydrate conjugate of Applicants' invention actually induces Tn specific antibodies in non-human primates immunized therewith as can be seen from pages 4991 and 4992. Therefore, it is deemed that the specification clearly shows the efficacy of the claimed peptide-carbohydrate conjugate for treating cancers.

It is deemed that B as now defined is not indefinite since it is now defined as being a carbohydrate moiety consisting of a tumor antigen or a carbohydrate derivative

thereof which is deemed to be clearly supported by the specification and withdrawal of this ground of rejection is requested.

All of the claims have been rejected under 35 USC 103 as being obvious over the Chong et al patent for reasons of record or Chong et al taken in view of the Jondal patent. The Examiner is of the opinion that whether the synthetic carbohydrate used by Chong et al functions as T-cell epitopes is immaterial as the compound conjugate is a known conjugate. The Examiner refers to lines 27 to 32 of column 7 of Chong et al as disclosing that the synthetic glyco conjugate can be used to produce vaccines eliciting antibodies against proteins which vaccines can be used to induce immunity towards tumor cells. The Examiner is of the opinion that Applicants' arguments were not commensurate in scope with the original claim 29.

Applicants respectfully traverse this ground of rejection since it is deemed that new claim 29 is not rendered obvious by the Chong et al reference taken alone or in view of the Jondal patent. Claim 29 is now directed to a carbohydrate peptide conjugate which induces an antibody response against a carbohydrate tumor antigen and the said conjugate is not obvious in view of the combination of Chong et al taken alone or with Jondal.

The Chong et al patent discloses dendrimeric conjugates which combine only peptide T and B-epitopes and Chong et al does not disclose or suggest any synthetic conjugate wherein the B-epitope is included in a carbohydrate moiety. Beginning at line

65 of column 3 through line 67 of column 6, Chong et al exclusively discloses synthetic conjugates wherein B-cell epitope is only a peptide compound and particularly, one of the P1, P2 and P6 proteins from hemophilus influenza (Hiv). It is clear from various portions of Chong et al particularly, line 62 of column 4 wherein B-cell epitipe of the P1 protein is cited in line 8 of column 5 wherein the B-cell epitope of P2 is cited and in line 19 of column 5 wherein B-cell epitope of P6 is cited.

When the PRP carbohydrate moiety is used in the dendrimeric structures of Chong et al, it is only as a carrier molecule as expressed by the term "PRP-carrier conjugate vaccine" in lines 50 to 51 of column 6. Moreover, even in the case where the PRP carbohydrate moiety included in several embodiments of the dendrimeric structures of Chong et al would induce some antibody response against PRP which is neither described nor taught in Chong et al. It would remain that the said antibody response would not consist an antibody response against the "carbohydrate tumor antigens"/

The Jondal teachings do not overcome the deficiencies of the Chong et al patent since Jondal is exclusively interested in raising a cyctotoxic T-cell response against a carbohydrate moiety and teaches exclusively a peptide-carbohydrate conjugate that raises a CTL response. One skilled in the art would have found absolutely no motivation to use any of the carbohydrate moieties disclosed by Jondal in the dendrimeric structures disclosed by Chong et al because he would not have foreseen that an effective antibody

response might be raised against carbohydrate moieties. Therefore, the prior art taken alone or in any combination would not suggest Applicants' invention to one skilled in the art and withdrawal of this ground of rejection is requested.

In view of the amendments to the claims and the above remarks, it is believed that the claims clearly point out Applicants' patentable contribution and favorable reconsideration of the application is requested.

Respectfully submitted,
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CAM:ds
Enclosures

[CANCER RESEARCH 59, 1520–1524, April 1, 1999]

# A Fully Synthetic Immunogen Carrying a Carcinoma-associated Carbohydrate for Active Specific Immunotherapy[1]

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## ABSTRACT

**Aberrant glycosylation of mucins leads to the exposure of cryptic carbohydrate antigens at the surface of carcinoma cells, which, therefore, represent potent targets for anticancer therapeutic vaccines. To date, the development of immunogens to stimulate immune response to such saccharidic antigens is based on carbohydrate conjugation to carrier proteins. However, these traditional protein conjugates are poorly defined in chemical composition and structure. As an alternative, we synthesized a multiple antigenic *O*-linked glycopeptide (MAG) carrying the carbohydrate Tn antigen associated with a CD4$^+$ T-cell epitope (MAG:Tn-PV). This fully synthetic immunogen is highly defined in composition and carries a high saccharidic epitope ratio over the entire molecule. The MAG:Tn-PV was able to induce anti-Tn IgG antibodies that recognize human tumor cell lines. A therapeutic immunization protocol performed with this fully synthetic immunogen increased the survival of tumor-bearing mice. Thus, the accurately defined and versatile MAG system represents an efficient strategy to induce carbohydrate-specific antitumor immune responses but may also be applicable to the prevention of infectious diseases, if it is based on bacterial oligosaccharides.**

## INTRODUCTION

T and Tn carbohydrate epitopes are found not only on a variety of epithelial cells derived from breast, pancreatic, and colon cancers but also on T lymphoma cells (1). These carbohydrate antigens are relevant markers for cancer diagnostic and prognosis, but they also represent potent targets for antitumor immune responses (2). Targeting immune responses to such truncated variants of glycan chains expressed by tumor cells represents an important goal for the development of antigen-specific therapeutic vaccines against cancers (3). Recent advances in the total synthesis of oligosaccharides expressed by tumor cells (4, 5) open new possibilities for the development of synthetic carbohydrate-based vaccines. The implication of carbohydrate antigens in the metastatic process of tumor cells also makes these antigens relevant targets for the prevention of metastasis and recurrence of cancers (6). Active antitumor immunization with an immunogen bearing the carbohydrate tumor markers may represent an alternative to conventional cancer therapy.

For immunization purposes, carbohydrates are traditionally conjugated to a carrier protein. Although this approach has proved to be successful (2, 7, 8), it has major limitations, as follows: (*a*) hapten-carrier systems can be subjected to carrier-induced suppression of the immune response directed against the haptenic molecule (9, 10); (*b*) the low molecular excess of the antigen over the carrier results in a small level of the desired antibodies compared to the total amount of antibodies produced; and (*c*) the protein conjugates present ambiguity in both composition and structure, which is a major obstacle for reproducible preparations. For these reasons, a fully synthetic immunogen, without protein carrier, could be of great interest in the development of a carbohydrate-based vaccine. Here, we tested the potential use of MAGs[3] (11) as synthetic alternative immunogens. This system presents a high density of the carbohydrate antigen at the surface of a minor oligomeric inert lysine core. As a result, the immune response only focuses on the tumor antigen, thus limiting irrelevant antibody production. Moreover, such synthetic conjugates are particularly attractive for both their purity and accurate chemical definition. These features are, indeed, essential for quality control and consistent batch-to-batch vaccine production. The glycopeptide derivatives are synthesized by well-known standard solid-phase peptide synthesis methodology, which can be easily automated.

## MATERIALS AND METHODS

**Syntheses.** The Tn antigens (α-GalNAc-Ser/Thr) were synthesized by classical methods (12, 13). Syntheses of the MAG:Tn-PV, MAP:PV, Tn-PV, and PV were performed by the solid-phase methodology using the FMOC chemistry, as described previously (11). After attachment of the β-alanyl spacer to the Wang resin, the lysine core was assembled by coupling successively two levels of FMOC-Lys-(FMOC)OH, providing four amino groups. The lysine core was further elongated by the protected amino acids of the T-epitope sequence of the poliovirus (KLFAVWKITYKDT) to produce the MAP:PV. Ultimately, the α-GalNAc-Ser was incorporated to the four branches peptide which gave the MAG:Tn-PV construct after deprotection and cleavage from the resin, as reported previously (11). All of the final constructs were purified by reverse-phase high-performance liquid chromatography and were characterized by amino acid analysis and electrospray mass spectrometry. The $Tn_3$-TT glycopeptide [Ser(α-GalNAc)-Thr(α-GalNAc)-Thr(α-GalNAc)-QYI-KANSKFIGITEL] was prepared by incorporation, step by step, of the appropriate peracetyl-glycosylated FMOC-Ser/Thr in the peptide sequence using 2-(1H-Benzotriazole-1-y*P*)-1,1,3,3 tetramethyluronium tetrafluoroborate/*N*-Hydroxybenzotriazole (TBTU/HOBT) as the coupling reagent. Deacetylation of the sugar residue of the glycopeptide was achieved with a catalytic amount of sodium methoxide in methanol at pH 11. The crude product was purified by high-performance liquid chromatography (11) with a gradient from 10 to 35% and 14.74-min retention time. Electrospray mass spectrometry: 2623 (calculated, 2623.56). Amino acid analysis: Ala, 1 (1); Asp, 1.04 (1); Glu, 2.16 (2); Gly, 1.08 (1); Ile, 2.95 (3); Leu, 1.1 (1); Lys, 2.04 (2); Phe, 1.01 (1); Ser, 1.86 (2); Thr, 2.76 (3); and Tyr, 0.97 (1).

**T-Cell Stimulation.** The recognition of the poliovirus T-cell epitope contained in the different constructs was analyzed using a specific T-cell hybridoma and A20 cells as antigen-presenting cells, as described previously (14). T-cell hybridomas ($10^5$) were cultured with $10^5$ A20 cells in the presence of the indicated construct in RPMI 1640 supplemented with 10% FCS, antibiotics, L-glutamine, and mercaptoethanol. Interleukin 2 synthesis following recognition by the T-cell receptor of hybridoma T cells was assessed by the proliferation of the interleukin 2-dependent CTLL cell line using [$^3$H]thymidine.

**Mice and Reagents.** Six- to 8-week-old BALB/c, SJL/J, and DBA/2 mice were from Iffa Credo. DBA/1 mice were from the animal colony of the Pasteur Institute. The anti-Tn mAb MLS128 (15) was provided by Dr. H. Nakada (Kyoto Sangyo University, Japan). Tn was conjugated to chicken OVA (Tn-OVA) at an initial molar ratio of 4000:1 using glutaraldehyde, as described previously (11).

**ELISA and Flow Cytometry.** Mouse sera were tested for anti-Tn antibodies by ELISA using the synthetic glycopeptide $Tn_3$-TT or the parent

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[3] The abbreviations used are: MAG, multiple antigenic glycopeptide; MAP, multiple antigenic peptide; FMOC, *N*-(9-fluorenyl)methoxycarbonyl; mAb, monoclonal antibody; OVA, ovalbumin; a-OSM, asialo ovine submaxillary mucin; KLH, keyhole limpet hemocyanin.

peptide TT (YIKANSKIGITEL). Ninety-six-well microtiter plates (Nunc, Roskilde, Denmark) were coated with 0.1 μg of antigen per well in 50 mM carbonate buffer (pH 9.6) and incubated for 1 h at 37°C. After washing with PBS containing 0.1% Tween 20, the serially diluted sera in buffer (PBS plus 0.1% Tween 20–1% BSA) were added to the wells for 1 h at 37°C. Following three washes, wells were treated 1 h at 37°C using goat antimouse IgG or IgM peroxidase conjugate (Sigma Chemical Co., St. Louis, MO) and *O*-phenylenediamine-$H_2O_2$ was then added as substrate. Plates were read photometrically at $A_{492\,nm}$ in an ELISA auto-reader (Dynatech, Marnes la Coquette, France). The negative control consisted of naive mouse sera diluted 100-fold. ELISA antibody titers were determined by linear regression analysis plotting dilution *versus* $A_{492\,nm}$. The titers were calculated to be the $\log_{10}$ highest dilution, which gave twice the absorbance of normal mouse sera diluted 1:100. Titers were given as the arithmetic mean ± SD of the $\log_{10}$ titers.

Mouse sera were also tested at a 1:250 dilution by flow cytometry on Tn-expressing human tumor cell lines, Jurkat and LS180, and on the TA3/Ha murine cell line. Cells were first incubated for 30 min with sera at 4°C in PBS containing 5% FCS and 0.1% sodium azide and then with an antimouse IgM/IgG goat antibody conjugated to FITC (Sigma). One % paraformaldehyde-fixed cells were analyzed on a FACScan flow cytometer (Becton Dickinson, San Jose, CA).

**Antitumor Immunotherapy.** The murine mammary adenocarcinoma cell line, TA3/Ha was grown by passage on BALB/c mice. After i.p. administration of 1000 TA3/Ha cells, 6-week-old BALB/c mice were injected s.c. with 50 μg of MAG:Tn-PV or control MAP:PV construct with 1 mg of alum. Survival of treated and untreated mice was followed for 50 days. Statistical analysis of survival curves was performed with the Statview software (Abacus Concepts) using the log-rank test.

## RESULTS

**The Tn Antigen and the T-Cell Epitope Included into the MAG Retain Their Antigenicity.** As shown in Fig. 1, the MAG we synthesized is composed of a dendrimeric lysine core structure with four arms. Each arm is linked to a $CD4^+$ T cell epitope (PV peptide: KLFAVWKITYKDT sequence from the poliovirus type 1; Ref. 14) with a single α-*N*-acetylgalactosamine-serine residue (Tn) at the $NH_2$ terminus {MAG:Tn-PV: $[Ser(\alpha\text{-GalNAc})]_4$-$(PV)_4$-$K_2$-K-βAla; Ref. 11}.

The B-cell antigenicity of the Tn antigen included in the MAG: Tn-PV construct was analyzed using the MLS128 anti-Tn mAb, which was generated after immunization with the LS180 adenocarcinoma cell line derived from a colon cancer patient (15). Fig. 2*a* shows that the MLS128 mAb recognized the MAG:Tn-PV construct but not the parent construct devoid of the Tn antigen, MAP:PV [$(PV)_4$-$K_2$-K-βAla]. This recognition was in the same range as that observed not

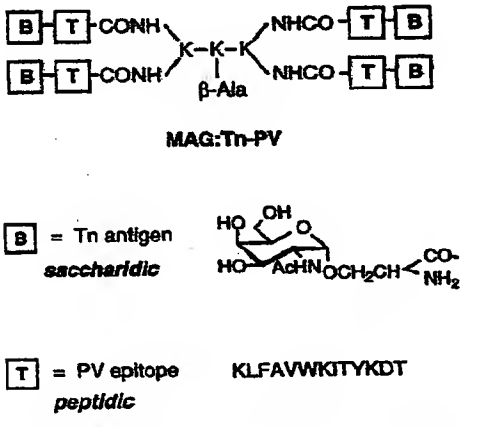


Fig. 1. Schematic representation of the MAG:Tn-PV molecule.

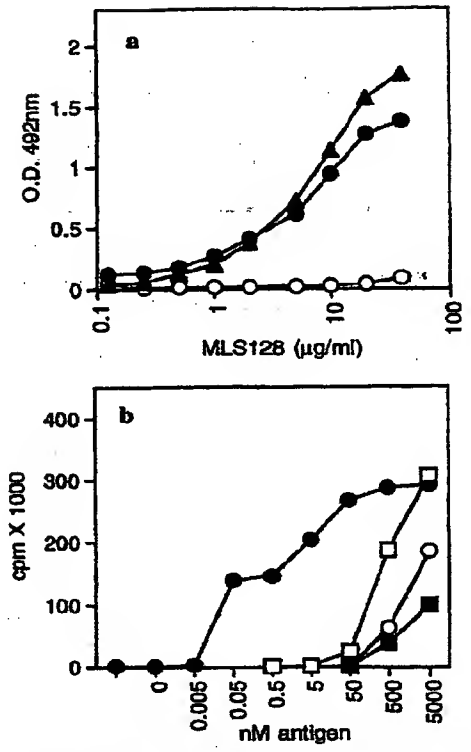


Fig. 2. B- and T-cell antigenicity of the MAG:Tn-PV. *a*, the MLS128 mAb specific for Tn was tested by ELISA for reactivity to Tn-OVA conjugate (▲), control MAP:PV (○), and MAG:Tn-PV (●) coated at 1 μg/mL. *b*, stimulation of a PV-specific T-cell hybridoma by the PV (□), Tn-PV (■), MAP:PV (○), and MAG:Tn-PV (●) constructs in the presence of A20 cells.

only with Tn conjugated to the chicken OVA protein (Fig. 2*a*) but also with the a-OSM (11).

The poliovirus-derived PV peptide was included in the MAG: Tn-PV to elicit a T cell-dependent antibody response. However, linkage of carbohydrates to peptides can interfere with peptide binding to MHC-encoded molecules and with T-cell recognition (16). For instance, Jensen *et al.* (17) showed that a T-cell hybridoma specific for a hemoglobin-derived peptide, glycosylated with α-GalNAc at a Thr residue, did not recognize the unglycosylated peptide *in vitro*. However, in this particular case, the glycosylation was introduced into the T-cell core sequence, whereas in our case, the Tn antigen was attached at the $NH_2$ terminus of the PV peptide (KLFAVWKITYKDT), clearly distant from the core sequence, which was characterized as FAVWKITYKD (14). As seen in Fig. 2*b*, a PV-specific T-cell hybridoma recognize the unglycosylated PV peptide as well as the glycosylated Tn-PV peptide showing that the Tn saccharidic epitope did not alter the T-cell recognition of the PV peptide sequence. To analyze the T-cell antigenicity of the MAG:Tn-PV, A20 antigen-presenting cells were incubated with the MAG:Tn-PV or the control constructs in the presence of a PV-specific T-cell hybridoma (Fig. 2*b*). It is noteworthy that the presence of the Tn antigen dramatically increased the presentation of the PV peptide by MHC molecules because T-cell stimulation was achieved with a 10,000-fold less MAG:Tn-PV dose compared to the MAP:PV construct and to the PV free peptide (Fig. 2*b*). This effect was only observed with the dendrimeric MAG structure because the stimulation induced by the Tn-PV glycopeptide [Ser(α-GalNAc)-PV] or the PV peptide was similar. The mechanisms underlying this enhancing effect remain

Table 1 *The MAG:Tn-PV-induced anti-Tn specific antibodies*[a]

| Mouse strain | Antigen | $Tn_3$-TT | | TT peptide | |
|---|---|---|---|---|---|
| | | IgM | IgG | IgM | IgG |
| BALB/c | MAP:PV | <250 | <250 | <250 | <250 |
| | MAG:Tn-PV | 6,660 ± 3,760 | 81,120 ± 18,000 | <250 | <250 |
| DBA/2 | MAP:PV | <250 | <250 | <250 | <250 |
| | MAG:Tn-PV | <250 | 6,020 ± 2,400 | <250 | <250 |
| SJL/J | MAP:PV | <250 | <250 | <250 | <250 |
| | MAG:Tn-PV | 800 ± 570 | 108,060 ± 34,600 | <250 | <250 |
| DBA/1 | MAP:PV | <250 | <250 | <250 | <250 |
| | MAG:Tn-PV | <250 | <250 | <250 | <250 |

[a] Mice (five per group) received three injections (days 0, 21, and 42) of 20 μg of MAP:PV or MAG:Tn-PV mixed with 1 mg of alum, except for DBA/2 mice, which only received two injections (days 0 and 21). Sera were collected 10 days after the last boost, and antibody titers specific for Tn were determined by ELISA using the $Tn_3$-TT glycopeptide or the parent TT peptide devoid of the Tn motifs. Results are expressed as the mean ± SE of individual antibody titers.

unclear. However, because the formation of stable peptide/MHC complexes requires intracellular peptide loading on MHC molecules, the enhanced PV peptide presentation observed with the MAG:Tn-PV may stem from the intracellular processing of this construct.[4] This result could also suggest that the MAG:Tn-PV endocytosis by antigen-presenting cells is mediated by the cross-linking of a GalNAc-specific receptor. After immunization of mice with the MAG:Tn-PV, T-cell responses specific for the PV epitope were stimulated *in vivo* (data not shown). Altogether, these results demonstrate that the Tn antigen on the MAG:Tn-PV construct is available for antibody binding and strongly enhances MHC presentation of the T-cell epitope, illustrating the potency of the MAG:Tn-PV construct to induce T cell-dependent anti-Tn antibodies.

**The MAG:Tn-PV Induces High Titers of Anti-Tn Antibodies That Recognize Tn-positive Tumor Cell Lines.** The PV peptide contains a promiscuous MHC binding sequence, which enables its presentation to T cells by I-$E^d$ and I-$A^s$ MHC molecules (14). Therefore, the immunogenicity of the MAG:Tn-PV was tested in different mouse strains expressing one of these MHC molecules. BALB/c (I-$E^d$), DBA/2 (I-$E^d$), and SJL/J (I-$A^s$) mice were immunized with the MAG:Tn-PV or with the control MAP:PV in alum, and sera were tested for anti-Tn antibodies (Table 1). The MLS128 mAb was shown to recognize three consecutive Tn antigens ([α-GalNAc]-Thr[α-GalNAc]) on a-OSM and glycophorin (18, 19). We, therefore, synthesized a glycopeptide, $Tn_3$-TT, irrelevant to the MAG:Tn-PV amino acid sequence containing these three Tn antigens at the $NH_2$ terminus of a linear peptide (TT) to evaluate by ELISA the level of anti-Tn antibodies. Immunization with the MAG:Tn-PV but not with the control MAP:PV induced anti-Tn IgG antibodies (mainly IgG1) in all three mouse strains tested. After three immunizations, Tn-specific IgM antibodies were still detected in BALB/c and SJL/J mice (Table 1). The Tn specificity of the antibodies using the $Tn_3$-TT glycopeptide was assessed by the lack of recognition by all mouse sera of the parent TT peptide devoid of the Tn antigen. DBA/1 (I-$A^q$) mice, which do not respond to the PV peptide (14), did not develop any anti-Tn antibodies following MAG:Tn-PV immunization, showing the T-cell dependency of the anti-Tn antibody response.

To ensure that mouse sera were able to recognize the native Tn antigen, we analyzed the binding of these sera to tumor cell lines expressing Tn. α-GalNAc-Ser/Thr is present on glycoproteins expressed by the human Jurkat T-lymphoma cell line (20) and LS180 adenocarcinoma cell line (15). Fig. 3, *a* and *b*, shows that anti-Tn positive sera from BALB/c mice primed with the MAG:Tn-PV bound both human cell lines as efficiently as the MLS128 mAb, whereas sera from naive mice or MAP:PV immunized mice did not recognize these cell lines. These results demonstrate that anti-Tn antibodies induced by the MAG:Tn-PV recognize the native form of Tn on human tumor cells.

**Immunotherapeutic Treatments with the MAG:Tn-PV Increase Mouse Survival and Resistance to Tumor Challenge.** A murine model has been developed to test active specific immunization against mucin-type carbohydrates using the TA3/Ha adenocarcinoma cell line (21). In this model, treatment with bovine- or ovine-desialylated mucin prior and after the tumor challenge afforded partial protection (22). Likewise, active immunotherapy in TA3/Ha-bearing mice using the T antigen β-Gal(1–3)α-GalNAc conjugated to the KLH protein together with cyclophosphamide was also able to strongly enhance mice survival (21). FACS analysis of the TA3/Ha cell line (which expresses the Tn antigen on mucin epiglycanin; Ref. 23) indicated that this cell line was recognized by MAG:Tn-PV induced antibodies (Fig. 3*c*). Therefore, we grafted 1000 TA3/Ha cells into BALB/c mice and followed their survival after active anti-Tn specific immunotherapy using the MAG:Tn-PV (Fig. 4). Following TA3/Ha graft, control groups of mice that were either left untreated (group 1) or treated with MAP:PV (group 2) displayed average survival times of 25 and 24 days, respectively, whereas after treatment with MAG:Tn-PV (group 3), the average survival time was delayed to 30 days for mice that did not reject the tumor. The TA3/Ha cell is originated from A mouse strain and was shown to grow on many allogeneic mouse strains, but its malignancy slightly varies from one mouse strain to an other, depending on the genetic background (24).

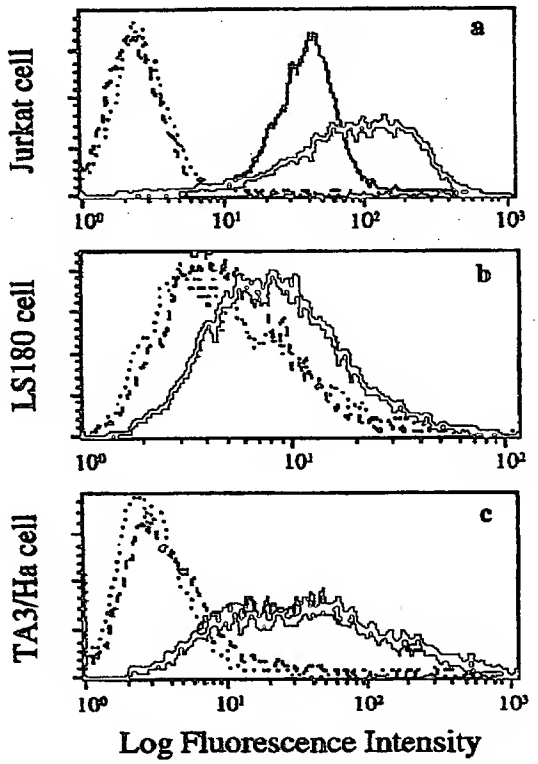


Fig. 3. Recognition of tumor cell lines bearing the Tn antigen by sera from MAG:Tn-PV-primed mice. Flow cytometry analysis was carried out on human Jurkat (*a*) and LS180 (*b*) cells and (*c*) murine TA3/Ha cells incubated with sera (diluted at 1:250) collected from BALB/c: naive mice (· · · ·), MAP:PV-primed mice (- - - -), MAG:Tn-PV primed mice (——) or the MLS128 mAb (——). Binding was detected using FITC labeled antibodies specific for mouse immunoglobulin. The positive staining observed with the serum from the MAG:Tn-PV primed mouse is representative of five individually tested sera.

[4] R. Lo-Man, unpublished data.

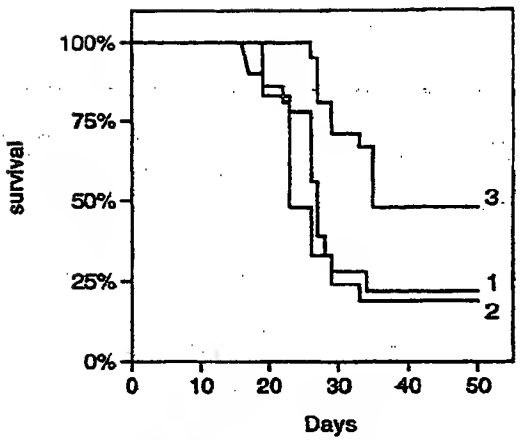


Fig. 4. Active specific immunotherapy in tumor-bearing mice. After i.p. administration of 1000 TA3/Ha adenocarcinoma cells (day 0), 6-week-old BALB/c mice were left untreated (group *1*) or received, on days 2, 5, 10, and 17, a 50-μg dose of the MAP:PV (group *2*) or the MAG:Tn-PV (group *3*) mixed with 1 mg of alum, and then mice were monitored for survival. Cumulative results of three independent experiments are presented corresponding to 18 mice in group 1 and 21 mice in groups 2 and 3. Differences are statistically significant between groups 1 and 3 ($P < 0.02$) and between groups 2 and 3 ($P < 0.01$).

Untreated BALB/c mice displayed a 10–20% rejection rate of the TA3/Ha after inoculation of $10^3$ cells showing that the graft of TA3/Ha was a little less efficient in BALB/c than in other mouse strains used in studies performed with the same tumor model (21, 22). However, statistically significant differences were observed in resistance or survival of mice following TA3/Ha implantation between group 3 (48%) and the control groups 1 (22%) and 2 (19%; $P < 0.02$). These data show that the anti-Tn immune response induced by the MAG:Tn-PV increases the survival of tumor-bearing mice by rejecting the tumor graft expressing the Tn carbohydrate antigen.

## DISCUSSION

Our study represents the first successful attempt to induce a strong immune response to a carbohydrate tumor marker using fully synthetic carbohydrate-based constructs as immunogens. Indeed, we show that the MAG:Tn-PV injected to mice can induce a strong IgG antibody response specific for the carcinoma-associated Tn antigen, and we demonstrate that this antibody response is T cell dependent. A previous attempt by Toyokuni *et al.* (3) to develop such a synthetic immunogen was based on the Tn antigen linked via a peptidic arm to a palmytoyl backbone. However, in this study, Tn-lipopeptides mainly induced an IgM antibody response specific for Tn rather than an IgG response, and this antibody response was T cell independent due to the lack of T-cell epitope in these lipopeptides. In the context of tumor associated carbohydrate antigens, the induction of a T cell-dependent IgG antibody response represents a main goal because human sera already contain natural IgM antibodies (1). In addition, it is striking to note that the presence of the Tn on the MAG:Tn-PV structure strongly enhances the level of MHC presentation of the PV T-cell epitope and probably contributes through enhanced T-cell activation to the high level of the IgG antibody response induced. The mechanism underlying this phenomenon is currently under investigation.

Our approach offers the advantages of a well-defined chemical structure and high purity, which are essential features for a safe vaccine, but it also provides a highly versatile peptidic core structure. Therefore, any carbohydrate moiety linked to an amino acid can be incorporated by standard solid-phase peptide synthesis methodology to always obtain the same carbohydrate content in the final compound. The glycopeptidic structure of the MAG construct may, thus, represent a future alternative to traditional protein conjugates carrying carcinoma or melanoma-associated carbohydrates that have been in clinical trials for several years (2, 7, 8, 25, 26). The MAG strategy may also represent an efficient way to induce antibodies specific for bacterial oligosaccharides because the induction of a T-cell dependent IgG response is also important to achieve, for instance, in the case of *Pneumococcus* vaccination in infants (27). Here, we did not compare the efficiency of our synthetic MAG construct to a carbohydrate-protein conjugate due to the paucity of qualitative and quantitative information on the latter. Moreover, such comparison remains difficult to achieve because the carbohydrate density on a carrier protein highly varies from one carrier protein to another and depends on many parameters, such as the conjugation method, the carbohydrate moiety, and the spacer arm that are used.

Our results demonstrate the potency of fully synthetic MAGs for active specific immunization, allowing, to a certain degree, the rejection of an implanted tumor-bearing aberrant glycosylations. Several hypothesis may explain the relative efficiency of antitumor immunity afforded by the MAG:Tn-PV treatment.

(*a*) On the basis of previous studies, total protection was never achieved in the TA3/Ha tumor model, and it seems that the immune mechanisms (humoral *versus* cellular response) that can lead to an efficient rejection of the TA3/Ha adenocarcinoma remain unclear (21, 22). Using a-OSM which expresses the Tn antigen, Singhal *et al.* (22) obtained 50% survival of TA3/Ha-bearing mice. In this study, immunization with a-OSM induced both an anti-Tn antibody response and an a-OSM-specific T-cell response. On the basis of putative sequence homology between OSM and TA3/Ha epiglycanin, these authors postulated that the T-cell response to glycopeptides carrying the Tn antigen could play a major role in the TA3/Ha rejection. Because the peptidic sequence of the MAG:Tn-PV is irrelevant to mucin-type protein sequences, clearly, MAG-treated mice did not benefit of such a T-cell response. Several studies demonstrated the ability of MHC molecules to bind synthetic glycopeptides to stimulate T-cell responses that are specific for the saccharidic moiety (16, 17, 28). But it remains to be established that the MHC presentation of glycopeptides naturally occurs following the intracellular processing of glycoproteins.

Fung *et al.* (21) using the T antigen conjugated to KLH mixed with RIBI and in combination with a cyclophosphamide treatment obtained a higher protection level in the TA3/Ha tumor model than we did in this study. However, it should be noticed that the Tn antigen is the precursor of the T antigen, and it can be suggested that upon deglycosylation both T and Tn antigens are available on KLH as a target for the immune system. This phenomenon could contribute to the efficiency of this conjugate. Moreover, the cyclophosphamide treatment plays a key role in the efficacy of the T-KLH conjugate-based immunotherapy and was not used in our experiments.

(*b*) The affinity and the diversity of Tn-specific antibody response induced by the MAG:Tn-PV molecule is not probably as high as it should be to allow a total clearance of the TA3/Ha tumor in mice. Indeed, the Tn antigen is clustered along the mucin protein sequence but not in the MAG:Tn-PV molecule. Indeed, the MLS128 mAb was shown to recognize a three consecutive Tn epitopes on a-OSM and glycophorin (18, 19). Because the linear Tn-PV glycopeptide with a single Tn antigen was not recognized by the MLS128 mAb (11) and did not induce any Tn antibodies (data not shown), the binding of the MLS128 mAb to the MAG:Tn-PV is probably due to the flexibility of the peptidic arms rebuilding a Tn cluster. Therefore, we can expect that a MAG carrying several Tn antigen in each arm will be more closely related to the multimeric Tn motif displayed by mucin pro-

teins. It also should be mentioned that multimerization of the Tn or the sialyl-Tn was shown to improve its immunogenicity when linked to a carrier protein (29, 30). Moreover, it was recently suggested for the sialyl-Tn antigen that carbohydrate clusters appear upon transformation of normal colonic tissues to malignancies (31). Therefore, it seems clear that the configuration of the carbohydrate epitope is at least as important as the total carbohydrate content to induce an optimal antitumor response.

Tested in clinical trials for many years now, classical carbohydrate protein conjugates are still under study to optimize their immunogenicity (32). The MAG molecule we tested here shows a high potency, but it only represents the first step of a new approach that needs to be much further developed, in particular by adding clustered carbohydrate epitopes, as mentioned above. Moreover, such immunogens probably would not focus on a single carbohydrate antigen but would, rather, combine various carbohydrate targets.

The use of a given Th cell epitope in conjunction with carbohydrates is a prerequisite for eliciting strong antibody responses, but this may limit the efficacy of the MAG immunogens considering the MHC polymorphism observed in the human population. To avoid this drawback, MAG structures have to include several T-cell epitopes with a particular focus on promiscuous MHC binding sequences, such as those described for tetanus toxin (33, 34), for which human individuals are already primed (35). Because dendrimeric peptide structures seem to be more efficient compared to linear peptide sequences to induce CTL responses (36), integration of CTL epitopes into MAG structures, such as MUC-1-derived peptides (37) for epithelial cancers, can also be achieved to widen the spectrum of the antitumor immune response. All these goals can be easily reached considering the versatility of the MAG system and can lead to a multicomponent therapeutic vaccine. Finally, it should be noticed that we have privileged the use of a mild adjuvant, alum, which is authorized in healthy human populations, showing that strong adjuvants are not required to induce anticarbohydrate specific immune responses by the MAG strategy. This latter point may be of major importance in extending the use of this strategy to bacterial oligosaccharides (27) for vaccinating a healthy population.

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# Short synthetic glycopeptides successfully induce antibody responses to carcinoma-associated Tn antigen

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**Abstract:** Glycopeptides contalning a tumor-associated carbohydrate antigen (mono-, tri- or hexa-Tn antigen) as a B-cell epitope and a $CD4^+$ T-cell epitope (PV: poliovirus or TT: tetanus toxin) were prepared for immunological studies. Several Tn antigen residues [FmocSer/Thr (α-GalNAc)-OH] were successively incorporated into the peptide sequence with unprotected carbohydrate groups. The tri- and hexa-Tn glycopeptides were recognized by MLS 128, a Tn-specific monoclonal antibody. The position of the tri-Tn motif in the peptide sequence and the peptide backbone itself do not alter its antigenicity. As demonstrated by both ELISA and FACS analysis, the glycopeptides Induced high titers of anti-Tn antibodies in mice, in the absence of a carrier molecule. In addition, the generated antibodies recognized the native Tn antigen on cancer cells. The antibody response obtained with a D-($Tn_3$)-PV glycopeptide containing three α-GalNAc-D-serine residues is similar that obtained with the $Tn_6$-PV glycopeptide. These results demonstrate that short synthetic glycopeptides are able to induce anticancer antibody responses.

**Abbreviations:** AgOTf, trimethylsilyl trifluoromethane sulfonate; $Ag_2CO_3$, silver carbonate; $AgClO_4$, silver perchlorate; a-OSM, asialo ovine submaxillary mucin; CFA, complete Freund's adjuvant; DIEA, diisopropylethylamine; ESMS, electrospray mass spectroscopy; FCS, fetal calf serum; FITC, fluorescein isothiocynate; Fmoc, 9-fluorenylmethoxycarbonyl; HOBT, 1-hydroxybenzotriazole; IFA, incomplete Freund's adjuvant; MAG, multiple antigen glycopeptide; MAP, multiple antigen peptide; OSA, ovine serum albumin; PE, phycoerythrin; PFA, paraformaldehyde; Pfp, pentafluorophenyl; PV, Poliovirus; TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; TT, Tetanus toxin.

As a result of aberrant glycosylation, cancer-associated carbohydrate antigens are exposed at the surface of some tumor cells, whereas they are shielded by further glycosylation or are poorly expressed in normal cells (1–3). Recent advances in immunology have renewed interest in the development of cancer vaccines, and these exposed glycosidic B-cell epitopes have been considered to be attractive targets for immunotherapy (4). Indeed, several studies have shown that efficient immune responses could be induced against different carbohydrate antigens: Tn (α-GalNAc-Ser/Thr), T (β-Gal-[1-3]-α-GalNAc-Ser/Thr), sialosyl-Tn (α-NeuAc-[2-6]-α-GalNAc-Ser/Thr) or the $GM_2$ ganglioside (5). The immunizations have usually been performed using carbohydrate–protein conjugates (6–8).

In order to circumvent the drawbacks displayed by protein carriers, the multiple antigen glycopeptide (MAG) approach was recently developed as an alternative to present carbohydrate antigens to the immune system (9, 10). This type of immunogen is based on the multiple antigen peptide (MAP) developed by Tam and co-workers (11, 12).

In a previous paper, we described the construction of a MAG carrying the carbohydrate Tn antigen (B epitope) associated with a $CD4^+$ T-cell epitope [PV: Poliovius (13)] on a dendrimeric lysine core with four branches (10). This MAG: Tn-PV was able to induce anti-Tn IgG antibodies that recognized human tumor cell lines. Moreover therapeutic immunization with this synthetic immunogen administered with alum increased survival in tumor-bearing mice (14).

The tumor-associated glycoprotein epitope defined by the monoclonal antibody MLS 128 is expressed on most human adenocarcinomas and is minimally expressed on normal tissues. MLS 128 was raised against LS 180 cells, a human colorectal cancer cell line (15). The epitope for MLS 128 has been identified as Tn, i.e. α-GalNAc, O-linked to Ser/Thr on mucin-type glycoprotein and it seems that a cluster of α-GalNAc-Ser/Thr is essential for Tn antigenicity (8, 16–18). In LS 180 cells, the Tn antigen has been shown to be the product of glycosylation of the polypeptide encoded by the *MUC*-2 gene (19). As a cluster, the Tn antigen has also been found in ovine submaxillary mucin (16), glycophorin (17) and the Jurkat cell lines (20).

In order to mimic the clustered motif encountered for the Tn antigen *in vivo*, further development of our MAG-based vaccines (10, 14) was undertaken, involving the introduction of a tri-Tn glycopeptide as the B epitope. Such an approach has already been described with di- and trimeric Tn conjugated to ovine serum albumin (OSA), Starburst dendrimers (18) and lipopeptides (21). The synthesis of linear glycopeptides (Fig. 1) was performed using Tn motifs associated with the $CD4^+$ T-cell epitope of the poliovirus (PV: KLFAVWKITYKDT). The Tn antigen was introduced in one, three or six copies and was located either in the middle or at the end of the peptide sequence. The antigenicity and immunogenicity of the resulting glycopeptides have been investigated and are described here. Since D-amino acids are known to confer proteolytic stability, D-($Tn_3$)-PV glycopeptide was synthetized in order to evaluate the effect of a D-amino acid on the antigenicity and immunogenicity of this peptide [D-($Tn_3$) represents three consecutive D-serine α-linked to a GalNAc residue]. Threonine was replaced in the tri-D-Tn motif by D-serine, since its β-chiral center might confer additional structural problems.

## Experimental Procedures

### Synthesis of the peptides and glycopeptides

The synthesis of the Tn antigens, appropriately protected, 9-fluorenylmethoxycarbonyl (Fmoc)Ser (α-GalNAc)-OH and FmocThr (α-GalNAc)-OH was performed using classical methods (22, 23) starting from tri-*O*-acetyl-D-galactal (24). *N*-(Fluorenylmethoxycarbonyl)-L-serine/threonine *tert*-butyl esters (25, 26) were used for the Koenigs-Knorr reaction with 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl chloride (27, 28). The catalyst used for the glycosylation was trimethylsilyl trifluoromethane sulfonate (AgOTf) for the L-amino acid (Ser, Thr) and silver carbonate ($Ag_2CO_3$)/silver perchlorate ($AgClO_4$) for the D-serine. The final deprotection of the acetyls (29) and the *t*-butyl ester afforded Tn antigens appropriately protected for the peptide synthesis.

| | |
|---|---|
| STTG$_6$KO | STTGGGGGGKG |
| Tn$_3$G$_6$KG | S*T*T*OGGGGGKO |
| Tn$_3$G$_6$K(Biot)G | S*T*T*GGGGGGK(Biotine)G |
| STTG6K(Biot)G | STTGGGGGGK(Biot)G |
| KO$_4$Tn$_3$G$_3$ | KGGGGS*T*T*GGG |
| PV | KLFAVWKITYKDT |
| Tn-PV | S*KLFAVWKITYKDT |
| Tn$_3$-PV | S*T*T*KLFAVWKITYKDT |
| D-(Tn$_3$)-PV | D-(S*)D-(S*)D-(S*)KLFAVWKITYKDT |
| Tn$_6$-PV | (S*T*T*G)$_2$KLFAVWKFITYKDT |
| Tn$_3$-TT | S*T*T*QYIKANSKFIGITEL |

*Figure 1.* Structure of the different glycopeptides. *, α-GalNAc; PV, poliovirus p 103–115; TT, tetanus toxin p 830–844; L-amino acids in single-letter code are designated by upper case letters, D-amino acids are specified by the letter D.

All the glycopeptides were assembled by the conventional solid-phase peptide methodology (Wang resin, Novabiochem) using Fmoc chemistry. The appropriately protected amino acids were incorporated manually into the peptide sequence using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBT)/diisopropylethylamine (DIEA) as the coupling reagent (30, 31). Fmoc protection was removed with 20% piperidine in DMF. The glycosylated amino acids FmocSer/Thr(α-GalNAc)-OH were incorporated as their pentafluorophenyl (Pfp) esters (32); these were prepared by addition of 1,3-diisopropylcarbodiimide to the glycosylated amino acid and pentafluorophenol in dry $CH_2Cl_2$ (33). The reaction was followed by TLC. After concentration of the solution the Pfp-esters were used without further purification and dissolved in DMF with HOBT, as described previously (33). The peptides were cleaved from the resin with aqueous trifluoroacetic acid (TFA; 95%).

All the derivatives were purified by HPLC using a Perkin–Elmer pump system with a UV detector at 230nm; the column was a Nucleosil $C_{18}$ (5 μm, 300A°, 250 × 10mm) and the gradient was performed with water (0.1‰ TFA)/acetonitrile over 20min. The peptides and glycopeptides (Fig. 1) were all characterized by amino acid analysis and mass spectrometry.

PV

HPLC: gradient from 0 to 65%, retention time 12.6min; FABMS: [MH⁺] 1613 (calcd 1611.9); amino acid analysis: Ala 1.16 (1), Asp 1.03 (1), Ile 0.96 (1), Leu 1.01 (1), Lys 2.92 (3), Phe 1 (1), Thr 1.84 (2), Tyr 0.99 (1), Val 0.94 (1).

Tn-PV

HPLC: gradient from 10 to 60%, retention time 11.2min; electrospray mass spectroscopy (ESMS): 1903 (calcd 1903.22); amino acid analysis: Ala 1 (1), Asp 1.05 (1), Ile 1.0 (1), Leu 1.05 (1), Lys 3.12 (3), Phe 1.05 (1), Ser 0.94 (1), Thr 1.97 (2), Tyr 1.06 (1), Val 1.0 (1).

$Tn_3$-PV

HPLC: gradient from 10 to 60%, retention time 10.3min; ESMS: 2512 (calcd 2511.8); amino acid analysis Ala 1 (1), Asp 1.05 (1), Ile 1.0 (1), Leu 1.05 (1), Lys 3.12 (3), Phe 1.05 (1), Ser 0.95 (1), Thr 3.74 (4), Tyr 1.06 (1), Val 1.0 (1).

D-($Tn_3$)-PV

HPLC: gradient from 15 to 30%, retention time 16.9min; ESMS: 2484 (calcd 2483.74); amino acid analysis Ala 1 (1), Asp 1.01 (1), Ile 0.96 (1), Leu 1.01 (1), Lys 3.06 (3), Phe 0.97 (1), Ser 2.7 (3), Thr 1.85 (2), Tyr 1.03 (1), Val 0.99 (1).

$Tn_3$-TT

HPLC: gradient from 10 to 35%, retention time 14.7 min; ESMS: 2623 (calcd 2623.56); amino acid analysis: Ala 1 (1), Asn 1.04 (1), Glu 2.16 (2), Gly 1.08 (1), Ile 2.95 (3), Leu 1.1 (1), Lys 2.04 (2), Phe 1.01 (1), Ser 1.86 (2), Thr 2.76 (3), Tyr 0.97 (1).

$Tn_6$-PV

HPLC: gradient from 10 to 60%, retention time 12.7min; ESMS: 3525.0 (calcd 3524.7); amino acid analysis: Ala 1 (1), Asp 0.98 (1), Ile 0.97 (1), Leu 1.0 (1), Lys 2.91 (3), Phe 1.02 (1), Ser 2.09 (2), Thr 5.92 (6), Tyr 1.13 (1), Val 1.0 (1).

$STTG_6KG$

HPLC gradient from 0 to 17%, retention time 8.2min; ESMS: 834.3 (calcd 834.4); amino acid analysis: Ser 0.93 (1), Thr 2 (2), Gly 7.95 (7), Lys 1.21 (1).

$STTG_6K$ (Biot)G

HPLC gradient from 0 to 20%, retention time 14.6min; ESMS: 1060.8 (calcd 1061.1); amino acid analysis: Ser 0.93 (1), Thr 1.89 (2), Gly 7 (7), Lys 1.09 (1).

$Tn_3G_6K$ (Biot)G

HPLC gradient from 5 to 20%, retention time 11.4min; ESMS: 1669.7 (calcd 1670.0); amino acid analysis: Ser 1.09 (1), Thr 2 (2), Gly 6.48 (7), Lys 0.99 (1).

$Tn_3G_6KG$

HPLC gradient from 0 to 17%; retention time 7.6min; ESMS: 1443.9 (calcd 1443.6); amino acid analysis: Ser 0.93 (1), Thr 1.79 (2), Gly 7 (7), Lys 1.0 (1).

$KG_4Tn_3G_3$

HPLC: gradient from 0 to 14%, retention time 9.6min; ESMS: 1444.4 (calcd 1443.6); amino acid analysis: Ser 0.88 (1), Thr 2 (2), Gly 7.07 (7), Lys 1.03 (1).

**Mice and immunization**

Six to 8-week-old female BALB/c mice were from Janvier (Le Genest Saint-Isle, France). Mice were injected intraperitoneally with peptides or glycopeptides in complete Freund's adjuvant (CFA; Sigma), then boost injections were performed in incomplete Freund's adjuvant (IFA; Sigma). Sera were collected and tested to detect for the presence of

anti-Tn antibodies by enzyme-linked immunosorbant assay (ELISA) or FACS.

#### ELISA (for antigenicity)

The PV- and TT-glycopeptides were coated in 50 mM carbonate buffer pH 9.6 by overnight incubation at 37°C on microtiter plates; several dilutions of MLS 128 [a mouse IgG3 specific for the Tn antigen (15)] were then added for 1 h at 37°C. After washing, the MLS 128 mAb bound to the coated compound was revealed using goat antimouse IgG peroxidase conjugate (Sigma) and *O*-phenyldiamine/$H_2O_2$ substrates. The reaction was stopped by $H_2SO_4$ and the optical density (OD) read at 492 nm with an ELISA autoreader (Dynatech, Marnes la Coquette, France).

For the inhibition studies, streptavidin-coated microtiter plates (Sigma, St Louis, MO, USA) were used and incubation with the biotinylated glycopeptide $Tn_3G_6K$ (Biot)G was performed for 1 h at 37°C. MLS 128 mAb, at 1 μg/mL, was then added to the streptavidin-coated plates with serial dilutions of the synthetic competitors (peptide or glycopeptide) for 30 min.

Results are expressed as the percentage of inhibition calculated from the values obtained without any competitor ($OD_{max}$) or in the presence of a competitor peptide ($OD_{comp.}$). % inhibition = $100 \times [1 - (OD_{comp.}/OD_{max})]$.

#### ELISA (antibody titer)

Mouse sera were tested for anti-Tn antibodies with ELISA using the synthetic glycopeptide $Tn_3G_6K$ (Biot)G or the unglycosylated analog $STTG_6K$ (Biot)G as control. The biotinylated peptides at 1 μg/mL were incubated for 1 h at 37°C on streptavidin-coated microtiter plates. Serial dilutions of sera were then added to the plates; bound antibodies were revealed using goat antimouse IgG or IgM peroxidase conjugate (Sigma). The titers were calculated to be the $\log_{10}$ highest dilution that gave twice the signal obtained with naive mice sera tested at a dilution of 1 : 100.

#### Flow cytometry (FACS)

Mouse sera were tested at serial dilutions by flow cytometry on the human Jurkat tumor cell line expressing Tn. Cells were first incubated for 30 min with sera at 4°C in phosphate-buffered saline (PBS) containing 5% fetal calf serum (FCS) and 0.05% sodium azide and further incubated 30 min with goat antimouse IgM conjugated to fluorescein isothiocyanate (FITC; Pharmigen, San Diego, CA, USA) and with a mixture of biotinylated antimouse IgG1, IgG2a, IgG2b and IgG3 antibodies from goat (Amersham, Les Ulis, France). IgG binding was then revealed using streptavidin-phycoerythrin (PE; Sera-Lab). Paraformaldehyde (PFA)-fixed cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analysis performed with CellQuest software (Becton Dickinson). The titers on Jurkat cells were calculated to be the $\log_{10}$ highest dilution that gave twice the geometric mean of fluorescence obtained with unstained cells.

## Results and Discussion

#### Synthesis

The glycopeptides were assembled by the conventional solid-phase peptide methodology using Fmoc chemistry, which is compatible with glycopeptide synthesis. The appropriately protected amino acids were incorporated into the peptide sequence using TBTU/HOBT/DIEA as the coupling reagent (30, 31). Of interest was the incorporation of three successive Tn residues, which could be achieved with the fully deprotected sugar. Several groups described the coupling of a single N-terminal glycosyl amino acid with unprotected carbohydrate hydroxyl groups (10, 34–36). However, to our knowledge, few examples of synthesis with subsequent coupling steps have been described (37–39). In our case three *O*-glycosylated Fmoc-amino acids Fmoc-Ser/Thr (α-GalNAc)-OH could be incorporated sequentially as their Pfp-esters (32, 33). This is very advantageous since it avoids potential side reactions (racemization and/or β-elimination) associated with the final deacetylation of the sugar residue, although this risk has been shown to be limited (40). Furthermore, deprotection of the acetyl groups of three sugar units is difficult to follow by HPLC when the structure is multimeric as in the case of the MAG constructs.

To synthesize the $Tn_6$-PV glycopeptide, a glycine residue was introduced as a spacer on the two amino groups of the N-terminal lysine residue to reduce steric hindrance (see Fig. 1 for the identification of the glycopeptides).

#### Antigenicity

The antigenicity of the mono-, tri- and hexa-Tn glycopeptides containing the PV sequence 103–115 (Tn-PV, $Tn_3$-PV, $Tn_6$-PV, respectively) and the tri-Tn glycopeptide with the TT sequence 830–844 ($Tn_3$-TT) was first evaluated by

measuring the recognition of the Tn motif using the MLS 128 mAb (Fig. 2A). The tri- and hexa-Tn peptides are efficiently recognized by MLS 128, whereas the mono-Tn-PV and the PV peptides are not. In contrast, the tri-Tn glycopeptide with three D-serine residues is also recognized by the antibody. Since the chirality of the D-($Tn_3$) motif in the PV-glycopeptide does not affect recognition of the 'Tn motif' by MLS 128, the aglycone (Ser/Thr) might not be of crucial importance.

The influence, on MLS 128 binding, of the position of the tri-Tn motif within the peptide backbone was then investigated. For this purpose, an inhibition assay was performed using $Tn_3G_6KG$ or $KG_4Tn_3G_3$ glycopeptides as competitors for the binding of MLS 128 to $Tn_3G_6K$ (Biot)G coated on a streptavidin layer. The model polyglycine glycopeptide $Tn_3G_6KG$ was chosen for the competition assays because it competes efficiently with asialo ovine submaxillary mucin (a-OSM) for MLS 128 binding, whereas the unglycosylated parent peptide, $STTG_6KG$, does not (data not shown). As can be seen in Fig. 2B, the inhibition is similar whether the tri-Tn cluster is at the N-terminal end of the peptide or in the middle of the peptide chain.

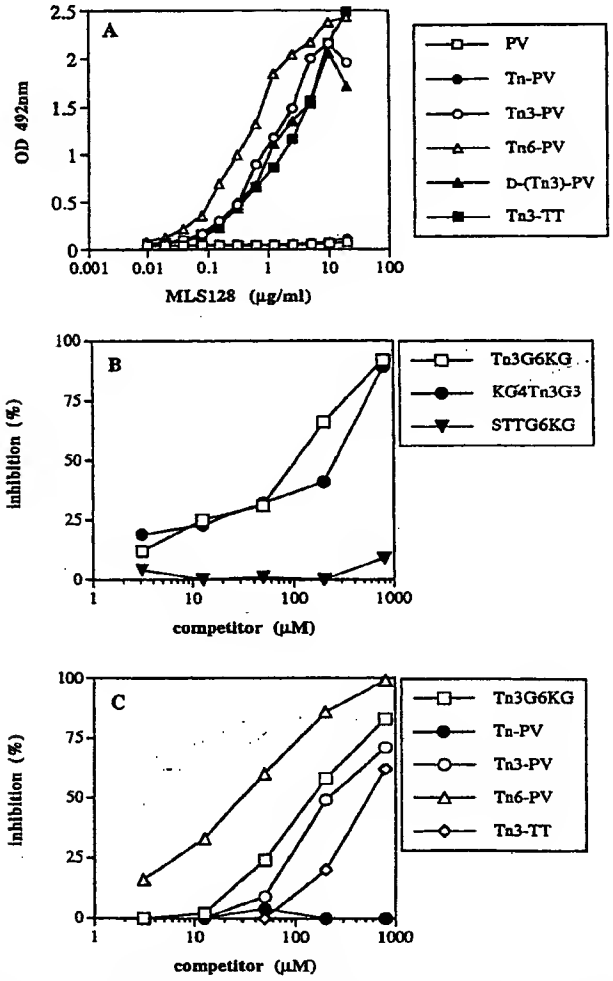


*Figure 2.* Antigenicity of Tn-glycopeptides using the MLS 128 mAb. MLS 128 binding to various Tn-glycopeptides was assessed by direct ELISA (A). Inhibition of MLS 128 binding to Tn3G6K(Biot)G (B, C) was performed by competitive ELISA using the indicated Tn-glycopeptides as described in Experimental Procedures.

Furthermore, recognition of the tri-Tn sequence by MLS 128 is affected only slightly by the nature of the adjacent peptide as shown in Fig. 2C, in inhibition studies with $Tn_3$-PV, $Tn_3$-TT or $Tn_3G_6KG$. These results indicate that the binding of the tri-Tn ligand to MLS 128 does not really depend on the peptide backbone. This is particularly important given the fact that the association of the Tn motif with a T-cell peptide is required for its immunogenicity.

Using MLS 128, strong immunoreactivity was observed with synthetic MUC-2 peptides (14 Thr) containing nine or 10 GalNAc residues; however, the site of glycosylation in the peptide was not clearly determined (41). In contrast, the MLS 128 mAb was shown to recognize a tri-Tn motif on glycophorin (17) suggesting that the density of the Tn motif can be a critical point for antibody recognition. This point is illustrated in Fig. 2C by the incapacity of a mono-Tn glycopeptide (Tn-PV) to inhibit MLS 128 binding.

In order to improve the antigenicity of the Tn-based glycopeptides, the density of the Tn motif was further increased by introducing one tri-Tn motif on each amino group of the N-terminal lysine residue of the PV peptide ($Tn_6$-PV). As shown in Fig. 2C, this strategy leads to significant enhancement of the antigenicity (measured in the competition assay) compared with the $Tn_3$-PV peptide.

### Immunogenicity

To analyze the ability of Tn-based linear glycopeptides to induce anti-Tn antibodies, BALB/c mice received three injections of the Tn-PV, $Tn_3$-PV, D-($Tn_3$)-PV and $Tn_6$-PV glycopeptides or of the control PV peptide corresponding to the T-cell epitope alone. Sera were collected after each immunization and tested for IgG and IgM anti-Tn antibodies by ELISA using a glycopeptide with a tri-Tn motif associated with a polyglycine backbone, $Tn_3G_6K$(Biot)G, which is unrelated to the PV sequence (Fig. 3). Another glycopeptide with an irrelevant sequence to PV was tested ($Tn_3$-TT) with similar results (unpublished data).

Under these conditions, anti-Tn IgM antibodies were detected after immunization with the $Tn_6$-PV peptide and D-($Tn_3$)-PV (Fig. 3A). A slightly lower antibody response was obained when mice were immunized with $Tn_3$-PV. Interestingly, these three short glycopeptides also induced

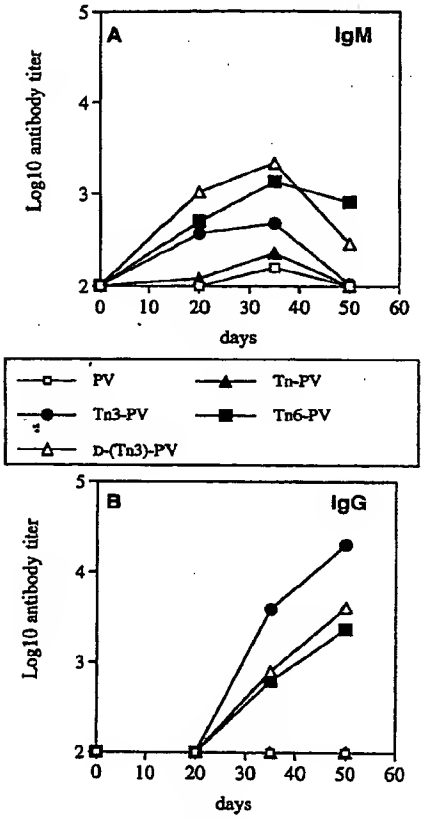


*Figure 3.* Anti-Tn antibodies induced in mice immunized with Tn-glycopeptides as measured by ELISA. Mice (four per group) received three injections (days 0, 21 and 42) of 50 μg PV, Tn-PV, $Tn_3$-PV, $Tn_6$-PV and D-$(Tn_3)$-PV in CFA for the first injection and in IFA for boost injections. After each injection, sera were collected and analyzed for anti-Tn antibody response using the $Tn_3G_6K$(Biot)G glycopeptide (IgM in A and IgG in B). No sera binding was observed in ELISA using the unglycosylated $STTG_6K$(Biot)G peptide as the coating antigen (data not shown). Results are expressed as the mean of individual antibody titers. Serum titers < 100 are considered negative since sera were tested at a starting dilution of 1 : 100.

anti-Tn IgG antibodies; unexpectedly however, the level was somewhat higher for the $Tn_3$-PV glycopeptide than for the D-$(Tn_3)$-PV and the $Tn_6$-PV homologs (Fig. 3B). This level, obtained with a linear glycopeptide in the absence of a carrier molecule, is similar to the IgG titer obtained with the dendrimeric MAG construct described earlier (10). We checked that no Tn-specific antibodies were detected after injection of the PV peptide showing the Tn specificity of the antibodies (Fig. 3B). Interestingly, the Tn-PV glycopeptide did not induce any Tn antibody. This result is in agreement with the lack of recognition of the mono-Tn glycopeptide by the MLS 128 mAb (Fig. 2C), and confirms that Tn cluster structures are required for efficient antibody production.

Peptides with contiguous B- and T epitopes have been reported to give a protective immune response in the absence of carrier molecules (42–45). However, little is known about the immunogenicity of glycopeptides as far as antibody production against the sugar moiety is concerned. Only a few examples of carbohydrate-specific antibodies raised after glycopeptide immunization have been published. Synthetic peptides containing either a pentasaccharide (46) or repeating units of 3-β-D-ribose-(1,1)-D-ribitol-5-phosphate (PRP) (47) have been shown to induce a good level of antibodies against the carbohydrate part of the glycopeptide. Other examples of immunogenic glycopeptides are known. However, the antibody specificity involves both the carbohydrate and peptide moieties (48, 49). Here we show that glycopeptides bearing a tri-Tn motif at the N-terminal end of a $CD4^+$ T-cell epitope are also able to induce the production of anti-Tn antibodies without the help of a carrier protein.

To determine whether these mouse sera were able to recognize the native Tn antigen, we titrated the anti-Tn reactivity obtained after the last immunization using human Jurkat T-lymphoma cells that express the Tn antigen (20). Figure 4 shows that sera from BALB/c mice immunized with $Tn_3$-PV, D-$(Tn_3)$-PV and $Tn_6$-PV bound this human cell line demonstrating that Tn-specific antibodies induced by these glycopeptides recognize the native form of

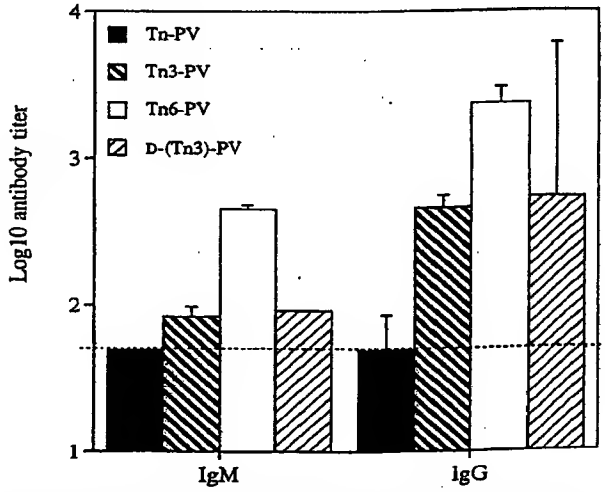


*Figure 4.* Anti-Tn antibodies induced in mice immunized with Tn-glycopeptides as measured by FACS using a human tumor cell line bearing the Tn antigen. Flow cytometry analysis was carried out on human Jurkat cells incubated with serial dilutions of sera from BALB/c mice (four per group) immunized on day 0 with 50 μg Tn-PV, $Tn_3$-PV, $Tn_6$-PV and D-$(Tn_3)$-PV in CFA, then boosted on days 21 and 42 in IFA with the same antigens. The sera are from mice bled 8 days after the last injection. Binding was detected using FITC-labeled antibodies specific for mouse IgM, and antimouse IgG biotinylated antibodies revealed with streptavidin-PE. Results are expressed as the mean of individual antibody titers calculated as indicated in Experimental Procedures. Serum titers < 50 (dashed line) are considered negative since sera were tested at a starting dilution of 1 : 50.

Tn on human tumor cells. However, the pattern of antibody titers is different whether the titration is performed by ELISA using the $Tn_3G_6K$ (Biot)G glycopeptide (Fig. 3) or by FACS using the Jurkat cell line (Fig. 4). Recognition of the Jurkat cells was better with $Tn_6$-PV- than with $Tn_3$-PV-induced antibodies (for both IgM and IgG). Interestingly, the antibody production obtained with the tri-D-($Tn_3$) glycopeptide [D-($Tn_3$)-PV] containing three D-serine residues is similar to that obtained with $Tn_3$-PV itself. This result could be useful for the development of MAG-constructs since it would protect the tri-Tn antigen from hydrolysis by the proteases.

The choice of the tri-Tn motif ($Tn_3G_6KG$) for the ELISA was based on the structural motif recognized by the MLS 128 mAb [17]. Although it allows the detection of antibodies raised against the $Tn_3$- or $Tn_6$-PV glycopeptides, it only represents a Tn artificial probe. By contrast, Tn recognition measured on Jurkat cells by FACS represents a more accurate view of the natural structure available at the cell surface for antibody recognition. Under these conditions, the double tri-Tn motif ($Tn_6$-PV) provides a significant benefit over the single tri-Tn motif ($Tn_3$-PV) in the induction of anti-Tn-specific IgM and IgG antibodies.

Although the Jurkat cell may not represent the cell prototype for the Tn expression level, no carcinoma cell clones can correctly mimic the natural diversity of mucinous cancer cells. Indeed, unveiling of cryptic Tn on carcinoma cells probably leads to highly variable Tn clusters on mucin-like proteins depending on the Ser/Thr content of the amino acid sequence. We may expect that if the Tn content is high in the glycopeptidic immunogen, the antibody response will be adapted for recognition of various types of natural Tn clusters.

In conclusion, we show here that the conjugation of tri- or hexa-Tn motifs to a linear $CD4^+$ T-cell epitope successfully induces anti-Tn antibodies. These results are promising for the further development of dendrimeric MAG constructs bearing tri- or hexa-Tn clusters. Indeed, a stronger response can be expected for these tri-Tn-MAG since the mono-Tn-MAG construct itself, when used in immunotherapy, has already been shown to give some protection in tumor-bearing mice [14].

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# Anti-Tumor Immunity Provided by a Synthetic Multiple Antigenic Glycopeptide Displaying a Tri-Tn Glycotope[1]

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**In many cancer cells the alteration of glycosylation processes leads to the expression of cryptic carbohydrate moieties, which make them good targets for immune intervention. Identification of cancer-associated glycotopes as well as progress in chemical synthesis have opened up the way for the development of fully synthetic immunogens that can induce anti-saccharide immune responses. Here, we synthesized a dendrimeric multiple antigenic glycopeptide (MAG) containing the Tn Ag *O*-linked to a CD4$^+$ T cell epitope. This MAG is based on three consecutive Tn moieties (tri-Tn) corresponding to the glycotope recognized by an mAb (MLS 128) produced against the LS180 colon carcinoma cell line. The Abs induced by this MAG recognized murine and human tumor cell lines expressing the Tn Ag. Prophylactic vaccination using MAG provided protection of mice against tumor challenge. When used in active specific immunotherapy, the MAG carrying the tri-Tn glycotope was much more efficient than the mono-Tn analogue in promoting the survival of tumor-bearing mice. Furthermore, in active specific immunotherapy, a linear glycopeptide carrying two copies of the tri-Tn glycotope was shown to be poorly efficient compared with the dendrimeric MAG. Therefore, both the clustering of carbohydrate Ags and the way they are displayed seem to be important parameters for stimulating efficient anti-saccharide immune responses.** ***The Journal of Immunology*****, 2001, 166: 2849–2854.**

The identification of tumor-associated Ags in melanomas and in several other human cancers has opened up a new basis for cancer vaccines (1). A number of tumor-derived peptides have been identified in the last few years and used as therapeutic immunogens in combination with different adjuvants, such as bacillus Calmette-Guérin, IFA, QS-21 (2, 3), or heat shock protein-peptide complexes (4). Other approaches based on DNA vaccines, recombinant virus shuttles, or tumor cells transfected with genes encoding costimulatory molecules or cytokines (5, 6) have also been developed to stimulate anti-tumor immunity. In the last few years, the use of dendritic cells has emerged as a new exciting area for cancer immunotherapy (7), because dendritic cells loaded with tumor peptides can induce immune responses preventing the outgrowth of tumors in mice (8). All these approaches aimed at stimulating peptide-specific T cell immune responses are promising; however, the efficacy of future immunotherapeutic treatments should rely on the stimulation of both Ab and cellular anti-tumor immune responses (9).

Tumor-associated carbohydrate Ags are also potential targets for anti-cancer therapy (10). Among these Ags, T, Tn, sialyl-Tn, and the ganglioside GM2 are the most well known. Covalent attachment of the carbohydrate Ags to protein carriers is traditionally used to induce anti-cancer Ab responses, and the resulting immunogens were shown to increase survival in patients. The most promising beneficial effects of tumor vaccination using this strategy were found for the GM2/keyhole limpet hemocyanin (KLH)[3] conjugate against melanoma (11) or the sialyl-Tn/KLH conjugate against breast, ovarian, and colorectal cancers (12).

Recently, we developed a fully synthetic immunogen that does not require protein carrier (13, 14). This system, called multiple antigenic glycopeptide (MAG), is based on a dendrimeric lysine core with four arms analogous to the multiple antigenic peptide (MAP) construct of Tam (15). Each arm is linked to a peptide backbone containing a CD4$^+$ T cell epitope (PV peptide) with a monomeric saccharide Tn residue at the N-terminal end of the peptide (MAG:Tn-PV). This construction offers several advantages: the carbohydrate content is much higher than in traditional protein conjugates, the core matrix is nonimmunogenic, and the construction has a well-defined chemical structure. A therapeutic immunization performed with this immunogen was shown to increase the survival of tumor-bearing mice (14).

Polysaccharidic cancer-associated Ags (GM2, GD2, Globo H, Le$^y$, Sialyl-Tn) can display large glycotopic structures available for Ab binding sites. In contrast, Tn, which is a monosaccharidic Ag (α-GalNAc-Ser/Thr) found on mucin-type glycoproteins and expressed on most human adenocarcinomas (16), was shown to be recognized by different mAbs as Tn clusters (17–20). Recent encouraging results obtained with a linear glycopeptide based on a tri-Tn glycotope recognized by the MLS128 mAb showed that anti-Tn Abs can be successfully induced in mice in the absence of a carrier protein (21). To further improve our MAG vaccines, the introduction of a cluster of three Tn was undertaken to mimic native sources of Tn on tumor cells.

We present here the results of both prophylactic and therapeutic vaccinations using a MAG construct with a lysine core carrying four copies of the PV peptide further extended with a tri-Tn glycotope (MAG:Tn3-PV). In both cases, the MAG:Tn3-PV afforded good protection against the development of Tn-expressing tumor cells.

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[3] Abbreviations used in this paper: KLH, keyhole limpet hemocyanin; MAG, multiple antigenic glycopeptide; ASI, active specific immunotherapy; CY, cyclophosphamide; MAP, multiple antigenic peptide; aOSM, asialo-ovine submaxillary mucin.



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## Materials and Methods

### *Synthesis*

The Tn Ags (α-GalNAc-Ser/Thr) were synthesized by classical methods (22, 23). Synthesis of the MAG:Tn-PV, MAG:Tn3-PV, MAP:PV, MAP:PV2, and Tn6-PV was performed by solid phase methodology using Fmoc chemistry as described previously (13, 21). After attachment of the β-alanyl spacer on the Wang resin the lysine core was assembled by coupling successively two levels of FmocLys-(Fmoc)-OH, providing four amino groups. The lysine core was further elongated by the amino acids of the T epitope sequence of the poliovirus (KLFAVWKITYKDT) (24) to produce the MAP:PV. The glycosylated amino acid α-GalNAc-Thr-OH was then introduced on the four branches as its pentafluorophenyl ester in the presence of *N*-hydroxybenzotriazole (21). The incorporation was repeated, and ultimately α-GalNAc-Ser-OH (pfp ester) was coupled to the construction. After deprotection and cleavage from the resin the products were purified by HPLC using a Perkin-Elmer pump system (Foster City, CA) with a UV detector at 230 nm; the column was a Nucleosil $C_{18}$ (5 μm, 300Å, 250 × 10 mm), and the gradient was performed with water (0.1% trifluoroacetic acid)/acetonitrile over 20 min (MAG:Tn3-PV: gradient from 5 to 60%; retention time, 12 min; amino acid analysis: Ala 3.75 (4), Asp 4.56 (4), Ile 4.13 (4), Leu 4.04 (4), Lys 16.6 (15), Phe 4.0 (4), Ser 3.82 (4), Thr 16.07 (16), Tyr 4.6 (4), Val 4.01 (4)).

### *Mice and Immunization*

Five- to 8-wk-old female BALB/c mice were obtained from Iffa Credo (St. Germain sur l'Abresle, France). To test immunogenicity of the MAG construct, mice were injected i.p. three times with Ag mixed with alum (Serva, Heidelberg, Germany) at 3-wk intervals. For vaccination experiments, mice received three i.p. injections of Ag mixed with alum or alum alone at 10-day intervals, and 10 days after the last boost, mice were challenged i.p. with 1,000 or 20,000 TA3/Ha cells prepared as described below. Alternatively, mice were challenged with the CT26 cell line (provided by Dr. R. A. Reisfeld, The Scripps Institute, La Jolla, CA) that does not express Tn. In some experiments, mice were treated with GK1.5 (anti-CD4) or H35.17.2 (anti-CD8) mAb (200 μg of mAb on days −1, 0, and 1 at the time of immunization). In all cases sera were collected after each immunization and tested for the presence of anti-Tn Abs by ELISA or FACS.

### *ELISA*

To test the binding of MLS128 (a mouse IgG3 specific for the Tn Ag (25); a gift from Dr. H. Nakada, Kyoto Sangyo University, Kyoto, Japan) to the MAG constructs, an ELISA was performed as previously described (13) by coating Ags at 1 μg/ml in 50 mM carbonate buffer, pH 9.6. Mouse sera were tested for anti-Tn as previously described (21), using the synthetic glycopeptide Tn3-G6K(Biot)G or the nonglycosylated analogue STTG6K(Biot)G as a control. Briefly, the biotinylated peptides at 1 μg/ml were incubated for 1 h at 37°C on streptavidin-coated microtiter plates. Then, serial dilutions of sera were performed, and bound Abs were revealed using goat anti-mouse IgG or IgM peroxidase conjugate (Sigma) and *o*-phenyldiamine/$H_2O_2$ substrates as previously described (13). Plates were read photometrically at 492 nm in an ELISA autoreader (Dynatech, Marnes la Coquette, France). The negative control consisted of naive mouse sera diluted 100-fold. ELISA Ab titers were determined by linear regression analysis, plotting dilution vs absorbance at 492 nm. The titers were calculated to be the $\log_{10}$ highest dilution that gave twice the absorbance of normal mouse sera diluted 1/100. Titers were given as the arithmetic mean ± SD of the $\log_{10}$ titers.

### *Flow cytometry*

Mouse sera were tested at serial dilutions by flow cytometry on two tumor cell lines expressing the Tn Ag, the human Jurkat cell (26), and the murine TA3/Ha cell (27). Cells were first incubated for 30 min with serial dilutions of sera at 4°C in PBS containing 5% FCS and 0.05% sodium azide. Then, cells were incubated 30 min with anti-mouse IgG conjugated to FITC and with an anti-mouse IgM conjugated to PE (Caltag, Burlingame, CA). Cells were analyzed on a FACScan flow cytometer (Becton Dickinson, Mountain View, CA), and analysis was performed with CellQuest software (Becton Dickinson). The titers were calculated to be the $\log_{10}$ highest dilution of sera that gave twice the geometric mean of fluorescence obtained with cells incubated with secondary reagents alone. For competition assay, $5 \times 10^5$ Jurkat cells were incubated with 1 μg/ml of the MLS128 mAb mixed with serial dilution of MAG, control MAP, or asialo-ovine submaxillary mucin (aOSM; given by Dr. E. Osinaga, Facultad de Medicina, Montevideo, Uruguay) for 30 min at 4°C, then the binding of MLS128 to Jurkat cells was revealed with an anti-IgG-FITC. Results are expressed as the percent inhibition of the signal obtained with MLS128 alone.

### *Anti-tumor immunotherapy*

For tumor implantation, the preparation of the murine mammary adenocarcinoma cell line, TA3/Ha, differs from our previously published work in which freshly isolated TA3/Ha from in vivo passages was directly used (14). Here, the TA3/Ha cells were grown by passage on BALB/c mice and were frozen to obtain a homogeneous batch. Two or 3 days before tumor graft, cells were thawed and cultured in vitro in DMEM containing 5% FCS. Cells were then recovered and centrifuged at 500 rpm for 5 min. In these conditions, cells recovered from the pellet were homogeneous in size and included <50% dead cells. Cells were checked for Tn expression by FACS using the MLS128 mAb before implantation. One thousand TA3/Ha live cells were administered i.p. to 5-wk-old BALB/c mice, then mice were s.c. injected several times, alternatively at the tail base and in the neck, with 100 μg of MAG constructs with 1 mg of alum. Survival of treated and untreated mice was followed for >100 days. Statistical analysis of survival curves was performed with StatView software (Abacus Concepts, Berkeley, CA) using the log-rank test.

## Results and Discussion

### *Antigenicity of MAG:Tn3-PV*

We previously showed that a dendrimeric MAG containing a monosaccharidic Tn motif at the N terminus of its four peptidic arms (MAG:Tn-PV) was able to induce Tn-specific Abs in a T cell-dependent manner (14). As the Tn Ag is expressed as clusters in mucin proteins, it should be advantageous to mimic naturally occurring Tn structures displayed by cancer cells. Moreover, dimeric and trimeric carbohydrate epitopes associated with protein carrier have already been shown to be more effective in generating Ab response than the monomeric analogue (28, 29). Similarly, we showed, using short synthetic glycopeptides, that a Tn cluster was required to induce an efficient immune response (21). To improve our MAG, we have therefore introduced a Tn cluster corresponding to the glycotope recognized by the MLS128 mAb. The MLS128 is a Tn-specific mAb obtained after immunizing mice with the human carcinoma cell line LS180 (25), and this mAb was shown to bind to a tri-Tn cluster ((α-GalNAc)-Ser-(α-GalNAc)-Thr-(α-GalNAc)-Thr) on mucin-like proteins (17, 18). This mAb can also recognize a dimeric Tn within the (α-GalNAc)-Ser-(α-GalNAc)-Thr-Thr sequence, but with a much lower affinity (30). Although we previously showed that a linear glycopeptide based on three consecutive (α-GalNAc)-D-Ser was able to induce anti-Tn Abs (21), the lack of contribution of the aglyconic part of the structure (Ser or Thr residues) to Ab binding is not clearly established. Therefore, based on the initial (α-GalNAc)-Ser-(α-GalNAc)-Thr-(α-GalNAc)-Thr sequence described for MLS128 binding, we synthesized a dendrimeric MAG with four arms containing the PV $CD4^+$ T cell epitope (MAG:Tn3-PV; see Table I and Fig. 1). Two dendrimeric MAP controls were also synthesized comprising the

Table I. *List of compounds used in this study*

| Compound[a] | Peptidic Backbone: PV peptide (no. of copy) | Peptidic Backbone: Additional amino acid[b] | Glycosidic Moiety No. of Tn Copies: Per branch | Glycosidic Moiety No. of Tn Copies: Per compound |
|---|---|---|---|---|
| STT-G6K(Biot)G | – | – | – | – |
| Tn3-G6K(Biot)G | – | – | 3 | 3 |
| MAP:PV | 4 | – | – | – |
| MAP:PV2 | 4 | STT | – | – |
| MAG:Tn-PV | 4 | S* | 1 | 4 |
| MAG:Tn3-PV | 4 | S*T*T* | 3 | 12 |
| Tn6-PV | 1 | $(S^*T^*T^*G)_2$ | 3 | 6 |

[a] MAP and MAG refer to dendrimeric compounds based on the $(Lys)_2$-Lys-βAla core. The PV peptide corresponds to the KLFAVWKITYKDT poliovirus sequence. [b] Asterisks refer to glycosylated amino acids.

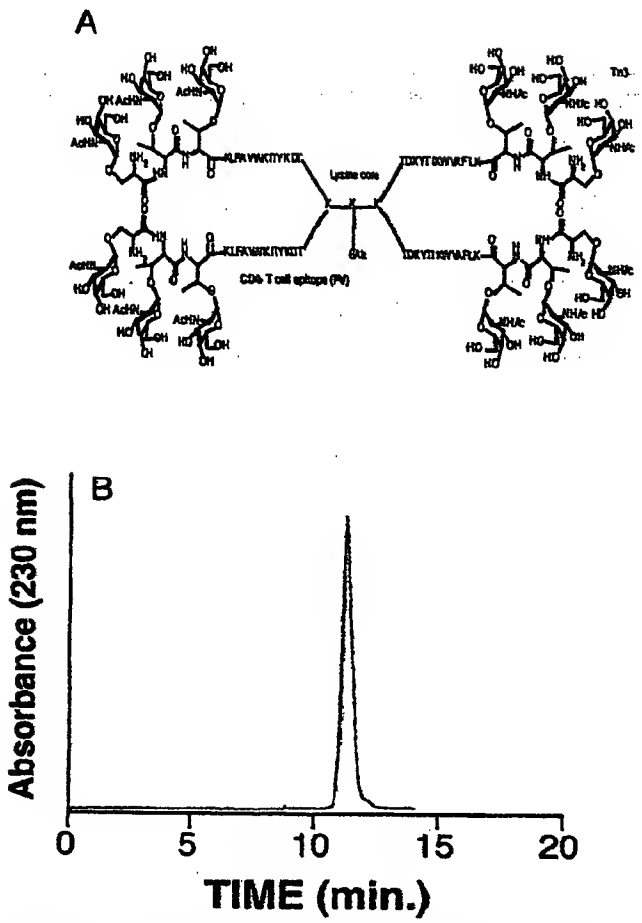


FIGURE 1. *A*, Schematic representation of MAG:Tn3-PV. *B*, HPLC elution profile of MAG:Tn3-PV.

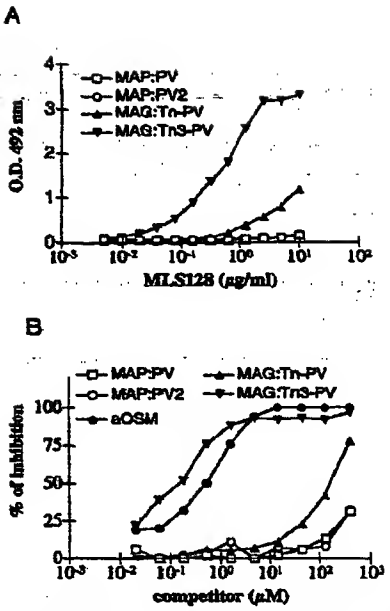


FIGURE 2. Compared antigenicity of mono-Tn- vs tri-Tn-based multiple antigenic glycopeptide. *A*, MLS128 mAb specific for Tn was tested by ELISA for reactivity to the MAG:Tn-PV, MAG:Tn3-PV, or control MAP:PV or MAP:PV2 coated at 1 μg/ml. *B*, Inhibition of MLS128 mAb binding to the Tn-positive Jurkat cell by MAG carrying the Tn Ag. Jurkat cells were incubated with MLS128 at 1 μg/ml together with various concentrations of MAG:Tn-PV, MAG:Tn3-PV, MAP:PV, MAP:PV2, or aOSM. MLS128 binding was detected by FACS using an anti-mouse IgG labeled with FITC. Results are expressed as the percent inhibition calculated from the Jurkat-associated fluorescence obtained with MLS128 alone.

PV sequence alone (MAP:PV) or the same construct with an additional STT peptide (MAP:PV2; Table I).

We first evaluated the recognition of Tn in the MAG:Tn3-PV by the MLS128 mAb. Using direct ELISA, the MLS128 Ab can very efficiently bind to the MAG:Tn3-PV compared with the former MAG:Tn-PV carrying a monomeric Tn (Fig. 2*A*). No MLS128 binding was observed using the control dendrimeric MAP compounds devoid of Tn (MAP:PV and MAP:PV2). To precisely compare the two MAGs, we tested by FACS the ability of the two constructs to inhibit the MLS128 binding to native Tn structures at the surface of the human Jurkat cell line. In these conditions, MAG:Tn3-PV strongly inhibited MLS128 binding to Jurkat cells, whereas MAG:Tn-PV did so only weakly (Fig. 2*B*). The MAG:Tn3-PV inhibition was as efficient as that obtained with aOSM.

*MAG:Tn3-PV induces Abs that recognize tumor cells*

We next evaluated the immunogenicity of the MAG:Tn3-PV compound by immunizing mice with MAG:Tn3-PV, MAG:Tn-PV or control MAP:PV2. Mice were immunized three times (days 0, 21, and 42) and were bled 1 wk after each immunization, and then regularly over a 6-mo period. Sera were tested for IgG and IgM Tn-specific Abs by ELISA using a biotinylated glycopeptide containing a tri-Tn cluster (Tn3-G6K(Biot)G). Fig. 3 shows that Tn-specific Abs (both IgG and IgM) were induced by MAG:Tn3-PV and MAG:Tn-PV, but not by the control MAP:PV2. However, the Tn-specific response induced by the MAG:Tn-PV slowly increased after immunization, whereas that induced by the MAG:Tn3-PV rapidly increased after two injections and then remained stable for 5 mo. The isotypic analysis of MAG:Tn3-PV-induced Abs shows that IgG1 Abs were predominant, but sera also contained a large amount of IgG3 Abs, which traditionally characterize anti-carbohydrate Ab responses in mice (data not shown). The increase in Tn density in the MAG did not modify the Th dependency of the anti-Tn Ab response, because this response was abolished when mice were depleted of CD4 T cells in vivo, but remained unchanged when CD8 T cells were depleted (data not shown). These results show that the introduction of a tri-Tn glycotope, instead of a monomeric Tn, into the MAG strongly increases the immunogenicity of the Tn Ag and makes the response more persistent.

Mucins and mucin-like proteins are characterized by a high degree of *O*-glycosylation through Ser or Thr residues; however, apomucin sequences and glycosylation frequency of acceptor sites remain heterogeneous. Recently, Reis et al. (20) showed that Tn-specific Ab responses induced in mice and rabbits by a glycosylated MUC2 peptide did not recognize Tn on asialo ovine submaxillary mucin, which is commonly used to test Tn reactivity. Therefore, the choice of a given Tn glycotope recognized by a single mAb, here three adjacent Tn on a STT backbone, could lead to the induction of a skewed anti-Tn immune response. To ensure that Tn-specific Abs induced by the MAG:Tn3-PV can recognize native Tn structures, we analyzed by flow cytometry the binding of sera from immune mice to tumor cells. As shown in Fig. 4, titration of sera from MAG:Tn3-PV-immunized mice on murine adenocarcinoma TA3/Ha cells demonstrates positive binding of both IgM

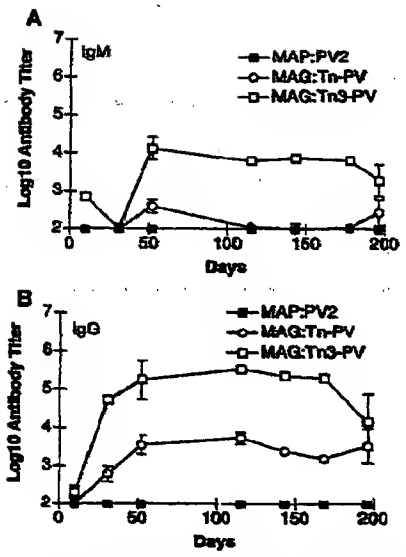


FIGURE 3. MAG:Tn3-PV induces a long-lasting Tn-specific Ab response. BALB/c mice (five per group) were immunized on days 0, 21, and 42 with 20 $\mu$g of MAP:PV2, MAG:Tn-PV, or MAG:Tn3-PV mixed with alum. Sera were collected 1 wk after each immunization or at various times until day 200 and were tested for Tn-specific IgM (*A*) and IgG (*B*) Abs by ELISA using Tn3-G6K(Biot)G.

and IgG Abs, as opposed to the lack of binding of sera from mice immunized with MAP:PV2. Likewise, the human Jurkat cell was well recognized by Abs induced after immunization with MAG: Tn3-PV, but not with MAP:PV2. These results show that native Tn structures on tumor cells are recognized by Abs induced by a MAG based on a tri-Tn glycotope, indicating that these Abs can efficiently target Tn-expressing tumor cells.

*Preventive vaccination with MAG affords protection against tumor challenge*

We next verified the capacity of MAG:Tn3-PV-induced Abs to target and reject the highly tumorigenic TA3/Ha adenocarcinoma in vivo. Mice were left untreated or were vaccinated three times with 10 $\mu$g of MAP:PV or MAG:Tn3-PV in alum or with alum alone. Then, 10 days after the last boost, mice were challenged with 1,000 TA3/Ha cells, and survival of mice to the TA3/Ha graft was followed for 100 days. As shown in Fig. 5*A*, all untreated mice died within 30 days following tumor challenge. In contrast, 80% of mice vaccinated three times with 10 $\mu$g of MAG:Tn3-PV mixed with alum survived to the tumor challenge, whereas 10% of mice survived in control groups receiving MAP:PV mixed with alum or alum alone (Fig. 5*A*). The protection afforded by MAG:Tn3-PV against the TA3/Ha challenge ranged from 70–90% depending on the dose of MAG used for vaccination (Fig.

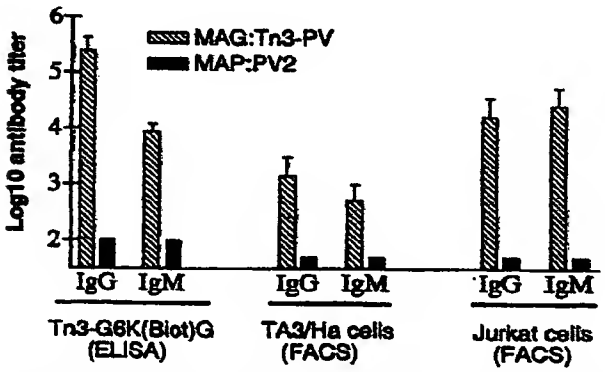


FIGURE 4. MAG:Tn3-PV-induced Abs bind to tumor cells expressing Tn. Serial dilutions of sera from mice (10/group) immunized on days 0, 21, and 42 with MAG:Tn3-PV or control MAP:PV2, as described in Fig. 2, were tested comparatively for binding to the Tn3-G6K(Biot)G by ELISA or to Tn-expressing tumor cell lines, the murine TA3/Ha cell or the human Jurkat cell, by FACS. Ig binding to tumor cells was revealed by double labeling with IgG-FITC and IgM-PE. Results are expressed as the mean of $\log_{10}$ individual Ab titers ± SD.

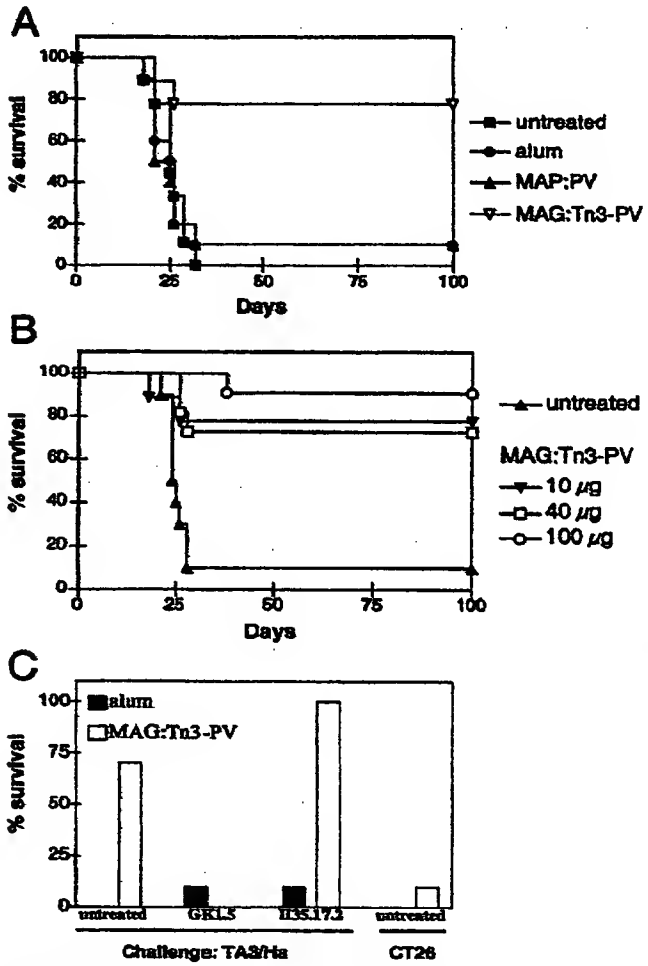


FIGURE 5. MAG:Tn3-PV vaccination protects mice against tumor challenge. *A*, Mice (10/group) were immunized on days −30, −20, and −10 with 10 $\mu$g of MAP:PV or MAG:Tn3-PV mixed with alum or with alum alone, or were left untreated. On day 0, mice received 1000 TA3/Ha tumor cells, and survival was followed for 100 days. Statistical analysis of the MAG:Tn3-PV-vaccinated group gave $p < 0.005$ vs untreated group, $p < 0.01$ vs alum-treated group, and $p < 0.01$ vs MAP:PV-treated group. *B*, Following the same schedule as that in *A*, mice (10/group) received three injections of 10, 40, or 100 $\mu$g of MAG:Tn3-PV with alum or were left untreated before being challenged with 1,000 TA3/Ha tumor cells. Mice were monitored for survival for 100 days after the tumor challenge. All MAG:Tn3-PV-vaccinated groups are statistically different from the control group ($p < 0.01$). *C*, Untreated, GK1.5 (anti-CD4)-treated, or H35.17.2 (anti-CD8)-treated mice were vaccinated with alum or with 10 $\mu$g of MAG:Tn3-PV mixed with alum and then challenged with 20,000 TA3/Ha cells or CT26 cells. Ten mice were used for each group, and survival was followed for 100 days.

5B). In contrast, MAG:Tn3-PV vaccination did not protect mice against the Tn-negative carcinoma CT26 cell line, showing the Tn specificity of the protection afforded by MAG. Prior to the tumor challenge, Tn-specific IgG were only detected in MAG:Tn3-PV-vaccinated mice (Ab titers ranging from 5,000 to 100,000 as detected by ELISA; data not shown). When mice were depleted of CD4 T cells in vivo, no anti-Tn Ab was induced, and the protection against TA3/Ha tumor challenge was totally abrogated, whereas CD8 T cell-depleted mice were still protected (Fig. 5C). Together, these results clearly show that the anti-Tn immune response induced by the MAG:Tn3-PV is able to confer a high protection rate against a tumor cell line expressing Tn, and that this protection depends on the induction of anti-Tn Abs requiring T cell help.

*MAG-based active specific immunotherapy (ASI)*

Preventive vaccination has limited interest when targeting the immune response to cancer-associated Ags; therefore, cancer vaccination should prove efficacious toward pre-existing tumor cells and be used as a therapeutic tool. Using the TA3/Ha tumor model, Fung et al. (31) showed that ASI treatments based on a β-Gal(1–3)α-GalNAc glycotope conjugated to the KLH (TF-KLH) can afford a substantial survival rate (25%) of TA3/Ha-bearing mice. Given the success obtained with the MAG:Tn3-PV vaccination on the survival of mice challenged with TA3/Ha cells, we next tested its efficacy in immunotherapeutic protocols. Mice were given 1000 TA3/Ha cells and were then treated with 100 μg of MAG:Tn3-PV in adjuvant (aluminum hydroxide or Freund's adjuvant) or with adjuvant alone. In these conditions, about 35–40% survival was observed in MAG:Tn3-PV-treated groups, whereas all mice died in groups treated with adjuvant or left untreated (Fig. 6A).

In various experimental models, anti-tumor immunity has been shown to be increased when immunotherapeutic treatments are combined with cyclophosphamide (CY) (32). The potentiation of anti-tumor immune response following CY treatment has been recently reported for both CD8 and CD4 T cell subsets, suggesting that CY treatments may indirectly contribute to the production of T cell growth factors (33, 34). Although the molecular basis of CY activity remains to be clarified, low doses of CY definitely increase delayed-type hypersensitivity and Ab responses. In the TA3/Ha tumor model, when a single dose of CY was given next to the TA3/Ha tumor graft, the efficiency of ASI based on TF-KLH or epiglycanin was highly increased, leading to a 50–90% survival rate of tumor-bearing mice (31). Likewise, a significant protection against TA3/Ha was also reported with a treatment combining CY and desialylated ovine or bovine submaxillary mucin (35). We therefore associated CY administration together with the MAG:Tn3-PV treatment to determine whether this could improve the efficacy of the ASI. Following TA3/Ha tumor implantation, mice received on day 1 a single dose of 10 or 50 mg/kg of CY. As shown in Fig. 6B, CY followed by immunization with alum alone did not modify the mortality of TA3/Ha tumor-bearing mice. When mice were given a 10 mg/kg dose of CY, followed by the MAG:Tn3-PV treatment given with alum on days 2, 5, 11, and 17, only 37.5% of mice survived. However, when MAG:Tn3-PV treatment was given after the administration of a 50 mg/kg dose of CY, the survival of TA3/Ha-bearing mice reached 80% (Fig. 6B). These results indicate that CY can improve the efficacy of the MAG:Tn3-PV treatment.

We further compared the efficacy of the MAG:Tn3-PV treatment with other immunogenic compounds. In a previous work, we showed

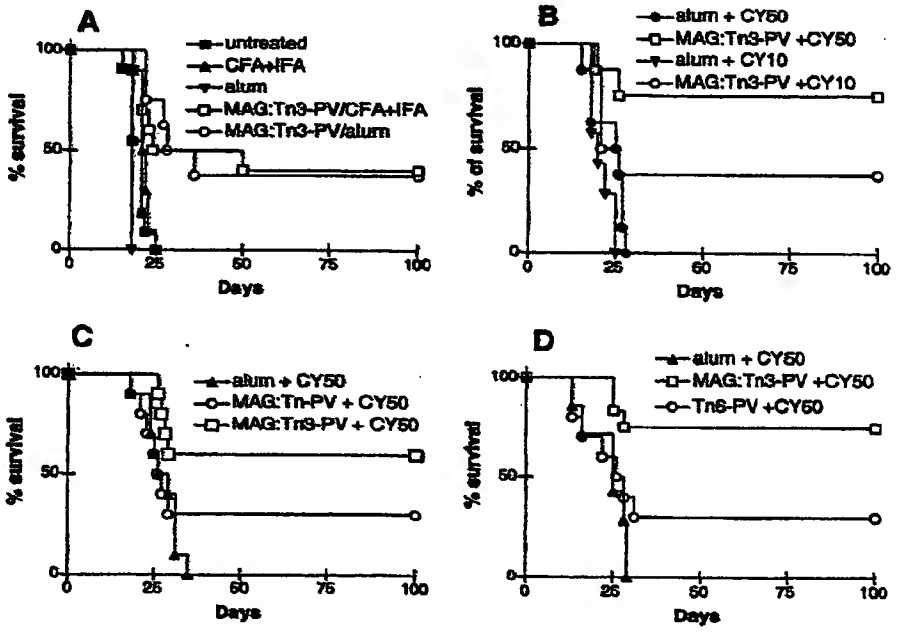


FIGURE 6. MAG-based immunotherapy successfully improved the survival of mice carrying a tumor expressing Tn. Mice received 1,000 TA3/Ha cells on day 0 and were then treated as described below or were left untreated, and survival was followed for 100 days. *A*, Mice (8–10/group) were treated with 100 μg of MAG:Tn3-PV in CFA/IFA or in alum or with adjuvant alone (CFA/IFA or alum) on days 2, 5, 11, and 17 ($p < 0.02$ between MAG:Tn3-PV/CFA and CFA groups and $p < 0.0001$ between MAG:Tn3-PV/alum and alum groups). *B*, Mice (8/group) received 10 or 50 mg/kg of CY on day 1 and then were treated with 100 μg of MAG:Tn3-PV in alum or with alum alone on days 2, 5, 11, and 17 ($p < 0.05$ between MAG:Tn3-PV/CY10 and alum/CY10 groups and $p < 0.005$ between MAG:Tn3-PV/CY50 and alum/CY50 groups). *C*, Mice (10/group) received 50 mg/kg of CY on day 1 and then were treated on days 2, 4, 6, 8, and 12 with alum alone or together with 100 μg of MAG:Tn3-PV or with 100 μg of MAG:Tn-PV ($p = 0.057$ between MAG:Tn-PV/CY50 and MAG:Tn3-PV/CY50 groups). *D*, Mice (7–12/group) received 50 mg/kg of CY on day 1, and then were treated on days 2, 5, 11, and 17 with alum alone or together with 100 μg of MAG:Tn3-PV or 100 μg of Tn6-PV ($p < 0.01$ between Tn6-PV/CY50 and MAG:Tn3-PV/CY50 groups).

that a MAG compound based on a monomeric Tn (MAG:Tn-PV) was able to improve the survival of TA3/Ha-bearing mice (14). Fig. 6*C* shows that ASI treatment performed with the MAG:Tn-PV together with CY leads to a 30% protection rate of tumor-bearing mice compared with the 60% protection afforded by the MAG:Tn3-PV-based ASI. This result is in agreement with the compared immunogenicity of both compounds (Fig. 3). More recently, we showed that a linear glycopeptide containing a T cell epitope associated with a double tri-Tn glycotope (Tn6-PV) was able to induce anti-Tn Abs that recognize Tn on tumor cell lines (21). The advantage of such a linear glycopeptide over the MAG strategy is that synthesis, yield, and purification are easier to achieve. When Tn6-PV was used in ASI, only 30% of mice survived to the TA3/Ha implantation compared with the 75% survival observed in the MAG:Tn3-PV-treated group (Fig. 6*D*). In summary, we show that the efficacy of ASI afforded by MAG is improved when the Tn Ag is incorporated as a tri-Tn cluster rather than as a mono-Tn moiety, and that the tri-Tn glycotope is much more efficient when displayed on the MAG structure compared with a linear glycopeptide.

It is difficult to ensure that the improved immunogenicity and therapeutic effects of the MAG:Tn3-PV compared with the MAG:Tn-PV result from the use of the tri-Tn glycotope and do not result from a clustering effect due to the higher amount of Tn incorporated into the immunogen. However, the results obtained with the linear glycopeptide (Tn6-PV) indicate that the beneficial effects of Tn clustering may be limited if the number of Tn Ag per branch is considered. Moreover, the injection of the same microgram doses of Tn6-PV and MAG:Tn3-PV corresponds to a 1.5-fold greater amount of Tn or tri-Tn in the case of the Tn6-PV, whereas it shows lower efficacy. Therefore, our results suggest that other parameters should be taken into account, for instance the flexibility of the saccharide moiety. Indeed, there is probably much more rigidity in the double tri-Tn cluster structure of the Tn6-PV, whereas the MAG can display a large variety of Tn glycotopes through its four flexible peptidic arms that can better mimic the diversity of natural Tn clusters. These issues are currently under investigation.

### *Conclusion*

Together, our results show that a synthetic multiple antigenic glycopeptide including a saccharide tumor-associated Ag together with an appropriate $CD4^+$ T cell epitope is highly immunogenic and can efficiently allow rejection of implanted tumor cells when used in therapeutic treatment. Our results also provide evidence that the introduction of well-defined glycotope clusters in carbohydrate-based immunogens is important for the induction of an efficient and long-lasting antitumor response.

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# Induction of carbohydrate-specific antibodies in HLA-DR transgenic mice by a synthetic glycopeptide: a potential anti cancer vaccine for human use

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**Key words:** glycopeptide; Pan-HLA-DR-binding epitope; synthetic vaccine for human use; Tn antigen; tumor-associated antigen

**Abstract:** Over the last few years, anticancer Immunotherapy has emerged as a new exciting area for controlling tumors. In particular, vaccination using synthetic tumor-associated antigens (TAA), such as carbohydrate antigens hold promise for generating a specific antitumor response by targeting the immune system to cancer cells. However, development of synthetic vaccines for human use is hampered by the extreme polymorphism of human leukocyte-associated antigens (HLA). In order to stimulate a T-cell dependent anticarbohydrate response, and to bypass the HLA polymorphism of the human population, we designed and synthesized a glycopeptide vaccine containing a cluster of a carbohydrate TAA B-cell epitope (Tn antigen: α-GalNAc-Ser) covalently linked to peptides corresponding to the Pan DR 'universal' T-helper epitope (PADRE) and to a cytotoxic T lymphocyte (CTL) epitope from the carcinoembryonic antigen (CEA). The immunogenicity of the construct was evaluated in outbred mice as well as in HLA transgenic mice (HLA-DR1, and HLA-DR4). A strong T-cell dependent antibody response specific for the Tn antigen was elicited in both outbred and HLA transgenic mice. The antibodies induced by the glycopeptide construct efficiently recognized a human tumor cell line underlying the biological relevance of the response. The rational design and synthesis of the glycopeptide construct presented herein, together with its efficacy to induce antibodies specific for native tumor carbohydrate antigens, demonstrate the potential of a such synthetic molecule as an anticancer vaccine candidate for human use.

**Abbreviations:** CEA, carcinoembryonic antigen; CTL, cytotoxic T lymphocyte; ESMS, electrospray mass spectroscopy; Fmoc, 9-fluorenylmethoxycarbonyl; HLA, human leukocyte-associated

antigen; RP-HPLC, reverse phase high-performance liquid chromatography; MAG, multiple antigenic glycopeptide; PADRE, Pan-HLA-DR-binding epitope; PV, poliovirus; TAA, tumor-associated antigen.

## Introduction

During carcinogenesis, modifications in the glycosylation processes lead to an altered carbohydrate pattern at the surface of cancer cells. This results in the expression of new tumor-associated antigens (TAA), some of which are correlated with a poor prognosis (1,2). Recent advances in the identification and synthesis of numerous TAA, together with a better understanding of tumor immunology, provide a strong basis for antitumor therapeutic vaccines designed to induce specific potent immune response against over-expressed carbohydrate TAA (3). Indeed, this strategy is a very active and promising field of research which holds tremendous potential as new and complementary approach for the treatment of cancer.

Amongst the identified TAA are some well-known carbohydrate antigens: Tn, T, sialyl-Tn, sialyl-T antigens, Lewis series antigens, and glycolipid-derived antigens (KH-1, globo H, fucosyl-$GM_1$, $GM_2$, $GD_2$, $GD_3$). Several of these TAA have been synthesized (3,4). The recent example of molecules associating several different carbohydrates TAA are of particular interest (5,6).

To trigger an efficient immune response, synthetic antigens are commonly linked to an immunogenic carrier protein. Immunization of mice with the resulting glycoconjugates elicit carbohydrate specific antibodies which sometimes recognize tumor cell lines (3,7–10). Some of the conjugates have been included in clinical trials for many years now (3,11,12). The antibody response was found to be associated with a beneficial clinical effect in some volunteers: $GM_2$-KLH, sialyl-Tn-KLH increase survival in patients with, respectively, melanoma (13) and breast cancers (14). However, many drawbacks are associated with the protein conjugates including carrier-induced epitopic suppression, low hapten density, irrelevant antibody production and uncertainty in both composition and structure. To overcome these limitations, we have recently developed totally synthetic epitope-based immunogens. Indeed, we synthesized glycopeptide vaccines displaying the Tn antigen (α-GalNAc-Ser/Thr) as the carbohydrate TAA (15). The Tn antigen is over-expressed on the membrane of epithelial tumors and is associated with many cancers including breast, lung, colon, prostate, and pancreatic cancers (16,17). Both linear and dendrimeric multiple antigenic glycopeptide (MAG) constructs successfully induced high titers of anti-Tn antibodies (IgM and IgG) that recognized tumor cell lines (18–20). When administered with alum, in either therapeutic or prophylactic protocol, these conjugates increased the survival of tumor-bearing mice (18,19). Among the advantages of these synthetic vaccines are their capacity to focus the immune response against the antigen of interest and their expected safety because of their well-defined chemical structure. However, the constructs we synthesized so far were designed for mouse immunization and therefore contain $CD4^+$ T-cell epitopes restricted to murine major histocompatibility complex (MHC) molecules.

To stimulate effective T-helper cell responses in human, Sette and co-workers engineered the Pan-HLA-DR-binding-epitope (PADRE) which is a synthetic non-natural T helper epitope selected for its capacity to bind to a wide range of HLA class II molecules (21). Linear peptides linked to PADRE were found to be potent immunogens *in vivo* (22–25). Furthermore, immunization with PADRE constructs encompassing B-cell epitope from the circumsporozoite protein of *Plasmodium yoelii* protected mice against *P. yoelii* sporozoite challenge (24). PADRE glycoconjugates also induced high titers of antibodies specific for oligosaccharide molecules (the lacto-*N*-fucopentaose II model and a dodecasaccharide from *Salmonella typhimurium*) (26).

In the present study, to further extend the scope of anticancer immunotherapy for human use, we designed a synthetic glycopeptide vaccine associating a cluster of the Tn carbohydrate tumor marker and the PADRE 'universal' T-helper epitope peptide. Although, this strategy does not provide cell immunity against TAA, it has the advantage to allow an effective population coverage. A cytotoxic T lymphocyte (CTL) epitope peptide derived from the tumor-associated carcinoembryonic antigen (CEA) (27) has also been introduced in order to improve the therapeutic efficacy of the vaccine through the induction of a cytoxic immune response. The immunogenicity of the resulting glycoconjugate vaccine was investigated in outbred mice as well as in HLA-DR-expressing transgenic mice. We found that (*i*) this totally synthetic glycopeptide construct induce carbohydrate-specific antibodies in both outbred and HLA-DR transgenic mice, (*ii*) the induced antibodies react *in vitro* with the native carbohydrate antigens expressed on the surface of human tumor cells, (*iii*) the PADRE provides efficient T-cell help for carbohydrate-specific antibody response in both types of mice.

## Experimental Procedures

### Synthesis of peptides and glycopeptides

The synthesis of the peptides and glycopeptides was performed as previously described by solid-phase peptide methodology using Fmoc chemistry (20). Briefly, the protected amino acids were incorporated manually into the peptide sequence using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBT)/diisopropylethylamine (DIEA) as the coupling reagent. Fmoc protection was removed with 20% piperidine in dimethylformamide (DMF). The glycosylated building blocks [Fmoc-Ser(α-GalNAc)-OH] were incorporated successively on the two branches of the lysine as their pentafluorophenyl (Pfp) esters in the presence of HOBT. The products were cleaved from the resin with aqueous trifluoroacetic acid (TFA), triisopropylsilane, $H_2O$, and phenol.

The peptides and glycopeptides were purified by reverse phase high-performance liquid chromatography (RP-HPLC) using a Perkin-Elmer (Courtaboeuf, France) pump system with a UV detector at 230 nm; the column was a Waters Delta Pak C18 (15 μ, 300 Å, 7.8 × 300 mm) and the gradient was performed with water (0.1% TFA)/acetonitrile over 20 min. The compounds were obtained with a purity from 85 to 99% as estimated by RP-HPLC using the Waters Delta Pak column or a Waters Symmetry column (C18, 5 μ, 300 Å, 3.9 × 150 mm). They were characterized by amino acid analysis and mass spectrometry. Mass spectra were recorded by electrospray in the positive mode on a Quattro-LCZ mass spectrometer (Micromass, Manchester, UK); the sample was dissolved at 10 mM concentration in water/acetonitrile (1/1) with 0.1% formic acid.

#### $Tn_6$-PAD

HPLC: gradient from 15 to 50%; retention time: 10.7 min.

Electrospray mass spectroscopy (ESMS): 4355.06 (calcd 4354.64); amino acid analysis: Ala 7 (7), Asn 2.29 (2), Gly 2.82 (3), Leu 4.72 (4), Lys 2.74 (3), Ser 5.63 (7), Thr 0.89 (1), Tyr 1.21 (1), Val 0.86 (1).

#### PAD

HPLC: gradient from 0 to 40%; retention time: 19.0 min.

ESMS: 3134.8 (calcd 3135.48); amino acid analysis: Ala 7 (7), Asn 2.15 (2), Gly 3.01 (3), Leu 4.26 (4), Lys 3.13 (3), Ser 5.94 (7), Thr 1 (1), Tyr 1.13 (1), Val 0.98 (1).

#### $Tn_3$-Pep

HPLC: gradient from 5 to 25%; retention time: 8.1 min.

ESMS: 1641.98 (calcd 1642.65); amino acid analysis: Gly 6.75 (7), Lys 1 (1), Ser 2.88 (3).

#### $Tn_6$-Pep

HPLC: gradient from 5 to 15%; retention time: 10.9 min.

ESMS: 2755.6 (calcd 2755.82), with a minor amount of $Tn_7$-Pep (M + 290); amino acid analysis: Gly 9 (9), Lys 2.13 (2), Ser 5.45 (6).

#### Mice

The OF1 outbred female mice, 6–8 weeks, were obtained from Charles River. Mice carrying DRA and DRB1*0101 (DR1) (28) or DRA and DRB1*0401 (DR4w4) (29) transgenes were kindly provided by Dr Dennis Zaller (Merck Research Laboratories, Rahway, NJ, USA). The transgenes encode chimeric human/mouse class II molecules in which the peptide-binding alpha 1 and beta 1 domains were derived from human DR sequences while the remainder of the molecule was derived from murine I-$E^d$ sequences. Thus, the mice generate DR-restricted T-cell responses upon peptide binding to the DR alpha 1 and beta 1 domains while the murine alpha 2 and beta 2 domains facilitate interaction with murine CD4. Transgenes were introduced into the B10.M/Sn strain because its endogenous class II molecule I-$A^f$ does not cross-pair with DR alpha or beta chains. Mice were confirmed to be transgene positive by polymerase chain reaction (PCR) analyses of tail DNA.

#### Immunization and analysis of antibody responses

The OF1 outbred mice were immunized intraperitoneally with either peptides or glycopeptides emulsified in complete Freund's adjuvant for the first injection and in incomplete Freund's adjuvant for boost injections. Immunization of HLA-DR1 and HLA-DR4 transgenic mice

was carried out by intraperitoneal injection of peptides or glycopeptides adsorbed to alum. Mouse sera were collected 10 days after the last boost, and tested for anti-Tn antibodies by an enzyme-linked immunosorbent assay (ELISA) using the synthetic glycopeptide Tn3-Pep or the non-glycosylated analogue Pep as a control (20). Briefly, the biotinylated peptides at 1 μg/mL were incubated for 1 h at 37 °C on streptavidin coated microtiter plates. Then, serial dilutions of sera were performed and bound antibodies were revealed using goat antimouse IgG or IgM peroxidase conjugate (Sigma-Aldrich, St Quentin, France) and *o*-phenyldiamine/$H_2O_2$ substrates as previously described (20). Plates were read at 492 nm in an ELISA auto-reader (Dynatech, Marnes la Coquette, France). The negative control consisted of naive mouse sera diluted 100-fold. ELISA antibody titers were determined by linear regression analysis, plotting dilution vs. absorbance at 492 nm. The titers were calculated to be the Log10 highest dilution, which gave twice the absorbance of normal mouse sera diluted 1/100. Titers were given for individual sera or as the arithmetic mean ± SD of the Log10 titers. Sera were also tested at serial dilutions by flow cytometry on a tumor cell line expressing the Tn antigen, the human Jurkat cell (30). Cells were first incubated for 30 min with serial dilutions of sera at 4 °C in phosphate-buffered saline (PBS) containing 5% foetal calf serum (FCS) and 0.05% sodium azide. Then, cells were incubated 30 min with antimouse IgG conjugated to fluorescein isothiocyanate (FITC) (Caltag, Burlingame, CA, USA). Cells were acquired on a FACScan® flow cytometer (Becton Dickinson, Mountain View, CA, USA) and analysis performed with the CellQuest software (Becton Dickinson).

## Results and Discussion

### Design and synthesis of the glycopeptide construct: covalent association of a carbohydrate B-cell epitope cluster to T-helper and CTL epitopes

We previously showed that linear and dendrimeric glycopeptides bearing the Tn TAA induce a strong anti-Tn antibody response in mice which is associated with a protection against tumor challenge (18). To mimic the native form of the antigen on tumor cells, we increased the Tn density on the vaccine construct and we showed that this provides a significant benefit in the induction of Tn-specific antibodies as well as in the protection against cancer (19). Most importantly, when BALB/c mice were immunized with a linear glycopeptide bearing a poliovirus (PV) T-helper cell epitope, the antibodies induced by Tn6-PV (with six copies of the Tn antigen) were found to recognize human tumor cells better than Tn3-PV-induced antibodies (20). Although linear glycopeptides are less efficient than MAG (19), they are easier to synthesize than complex dendrimeric structures. Therefore, they represent good models for evaluating the therapeutic potential of varions carbohydrate-based vaccines (20).

In order to obtain an immunogen capable of inducing anti-Tn antibodies in human and to overcome the extreme polymorphism of HLA class II molecules of the human population, we synthesized the linear glycopeptide Tn6-PAD as an anticancer vaccine model for human use (Fig. 1). Tn6-PAD is composed of (*i*) six copies of the carbohydrate Tn antigen (two tri-Tn glycotopes) which mimic the clustered occurrence of the antigen *in vivo*, (*ii*) the PADRE T-helper epitope (AKXVAAWTLKAAA, X = cyclohexyalanine), which binds to many murine and human MHC class II molecules and (*iii*) a CTL epitope (YLSGANLNL) from CEA which binds to the HLA-A0201 molecule. The CEA is expressed on several cancer types

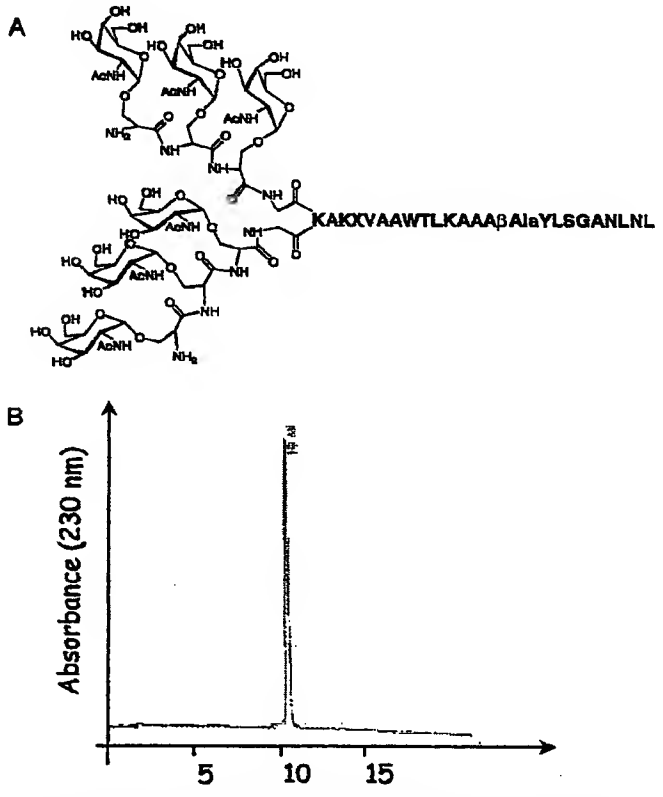


*Figure 1.* (A) Schematic presentation of the Tn6-PAD. (B) Reverse phase high-performance liquid chromatography (RP-HPLC) elution profile of Tn6-PAD. Chromatographic conditions: Waters Symmetry C18 (5 μ, 300 Å, 3.9 × 150 mm). Flow rate 1 mL/min. Gradient with water (0.1% trifluoroacetic acid (TFA)]/acetonitrile (15–50%) over 20 min.

(colorectal, gastric, breast, lung cancers) and the YLSGANLNL peptide has been shown to induce a cytoxic immune response against human adenocarcinoma cells (27).

Recently, synthesis and immunological evaluation of a glycopeptide bearing T-cell epitopes and a TAA was reported (31). The preliminary results showed that this glycopeptide seems to be able to induce a cytotoxic T-cell response.

The peptide and glycopeptides (Figs 1 and 2) were assembled by the conventional solid-phase peptide methodology using Fmoc chemistry. The CEA peptide was introduced in the C-terminal end and associated to the PADRE peptide using a β-alanine as a spacer. To synthesize the Tn6-glycopeptides, a glycine residue was introduced on each of the two amino groups (α and ε) of the N-terminal lysine residue to reduce steric hindrance between the two Tn3 motifs. The protected amino acids were incorporated into the peptide sequence using TBTU/HOBT (32). The *O*-glycosylated amino acids [Fmoc-Ser(α -GalNAc)-OH] were sequentially introduced at the N-terminal end as their Pfp ester (three cycles) (33), two copies of the same amino acid being added per cycle for the Tn6 peptides. These steps were performed with the fully deprotected sugar as described previously (15). Preliminary attempts with TBTU/HOBT activation were unsuccessful as a result of side reactions (data not shown). This strategy avoids a final deprotection of the carbohydrate residues which may give rise to secondary products. Furthermore, when multimeric carbohydrate structures are involved, deacetylation may be complex to follow by HPLC and some specific site deprotection difficult to achieve (34). Few examples of such incorporation of unprotected sugar residue have been reported with subsequent coupling of non-glycosylated amino acids (35,36). In our case, multiple unprotected glycosylated building blocks could be successively incorporated as their Pfp esters.

Glycopeptides with an irrelevant peptide (Tn3-Pep and Tn6-Pep) were synthesized to demonstrate the Tn specificity of the immune response (Fig. 2). The PAD and the Pep (20) compounds, devoid of Tn antigen, were also prepared as controls (Fig. 2).

During recent years, the synthesis of Tn clusters has been reported for subsequent incorporation in protein conjugates (37,38). Several groups have also synthesized numerous Tn containing glycopeptides including glycophorin, epithelial cadherin and, more importantly, mucin fragments [reviewed in (39,40)]. To our knowledge, the present study is the first report of a synthetic glycopeptide with a covalent association of three essential components for a anticancer vaccine: a T-helper epitope, a CTL epitope and a highly clustered carbohydrate TAA B-cell epitope.

| | |
|---|---|
| Tn3-Pep | S*S*S*GGGGGGK(Biot)G |
| Tn6-Pep | $(S^*S^*S^*G)_2$KGGGGGGK(Biot)G |
| PAD | $(SSSG)_2$KAKXVAAWTLKAAAA$^{\#}$YLSGANLNL |
| Tn6-PAD | $(S^*S^*S^*G)_2$KAKXVAAWTLKAAAA$^{\#}$YLSGANLNL |

*Figure* 2. Structure of the different synthetic (glyco)peptides. *: α-GalNAc; Biot: biotine; L-amino acids in single-letter code are designated by upper case letters; A$^{\#}$: β-alanine; X: cyclohexylalanine.

### Induction of carbohydrate-specific immune response in mice by the synthetic glycopeptide construct

The PADRE peptide AKXVAAWTLKAAA, incorporated in the Tn6-PAD glycopeptide, has been previously shown to bind HLA-DR1, DR4W4, DR4W14, DR5, and DR2W2A as well as a number of murine H-2 MHC II molecules (21). We first studied the immunogenicity of Tn6-PAD vs. the control PAD, in outbred $OF_1$ mice. Sera from immunized mice were tested by ELISA against Tn3-Pep or against a control peptide devoid of Tn (Pep). $OF_1$ mice immunized with Tn6-PAD, but not with PAD, developed Tn-specific IgG antibodies (Fig. 3A). The antibodies induced by Tn6-PAD are specific for the Tn antigen, as demonstrated in ELISA by the recognition of a Tn glycopeptide bearing an irrelevant peptide (Tn3-Pep) and by the absence of binding to the synthetic peptide Pep lacking Tn (Fig. 3A). To clearly assess the capacity of the Tn6-PAD glycopeptide to induce anti-Tn antibodies in an HLA restricted manner, we next investigated the immunogenicity of Tn6-PAD in mice transgenic for human class II molecules, HLA-DR1 and HLA-DR4. The Tn6-PAD was found to elicit anti-Tn antibodies (IgM and IgG) in both transgenic mouse strains (Fig. 3B,C). The Tn specificity of the antibodies was assessed by the recognition of glycopeptides with the irrelevant peptide Pep (Tn3-Pep and Tn6-Pep). PAD-specific T $CD4^+$ responses were also detected from immunized animals following proliferative response of peripheral blood T lymphocytes, confirming that the C-terminal addition of the CEA CTL epitope in Tn6-PAD did not affect the T-helper activity of the PADRE epitope (data not shown).

The cytotoxic immune response directed to the CEA epitope is currently under investigation.

### Biological relevance of the anticarbohydrate antibody response

To ensure that the Tn-specific antibodies induced by Tn6-PAD can recognize the native Tn antigen, we analyzed the

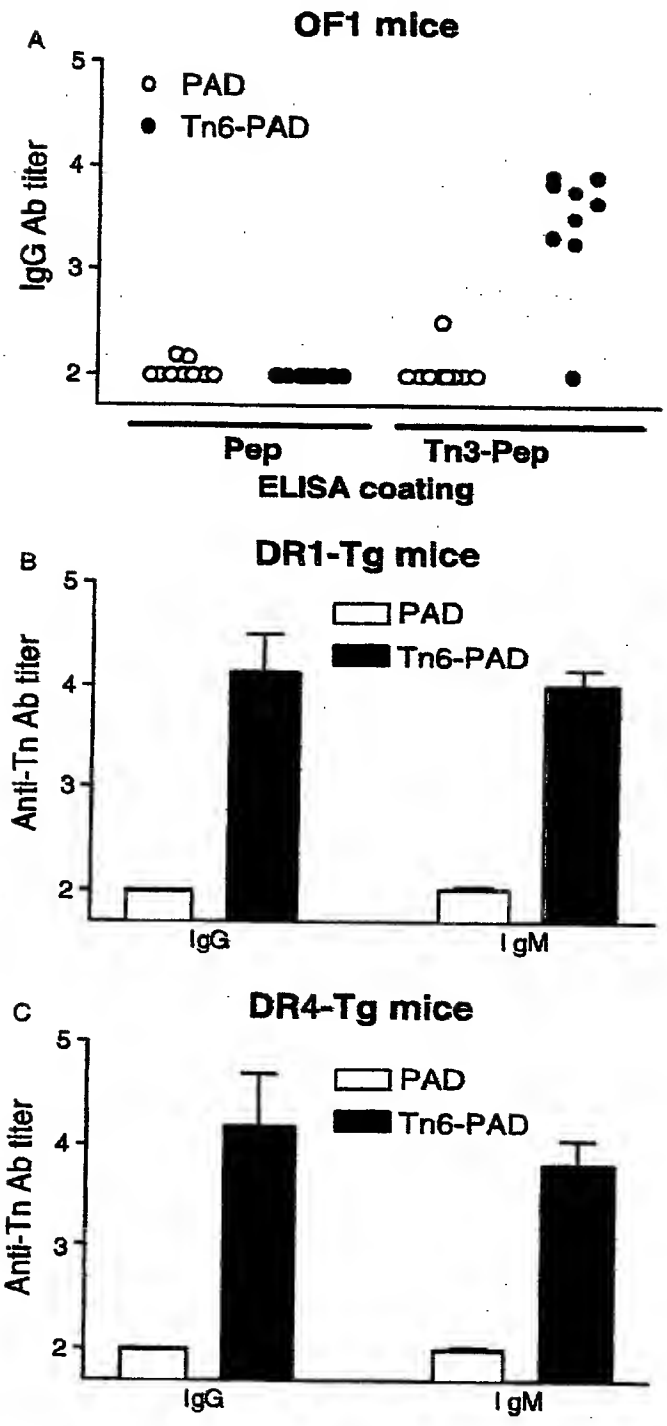


*Figure 3*. Immunogenicity of Tn6-PAD. Mice were immunized intraperitoneally, on days 0, 21 and 42, with 100 μg of Tn6-PAD glycopeptide or control PAD peptide in Freund's adjuvant (OF1 mice) or in alum (HLA-DR transgenic mice). The sera were collected 10 days after the last boost, and tested for anti-Tn antibodies by enzyme-linked immunosorbent assay (ELISA) using Tn3-Pep. (A) Experiments were performed in OF1 mice (A) or in HLA-DR1 (B) or -DR4 (C). In A, titers obtained with Pep control for coating show the specificity of the assay. IgG (A–C) and IgM (B–C) antibodies in the sera were tested. Results are expressed as individual antibody titers for nine mice (A) or as the mean ± SD of antibody titers of four mice per group (B and C).

The feasibility of using synthetic glycopeptides as vaccines has already been demonstrated. Indeed, several groups have reported efficient anticarbohydrate immune responses after immunization in animal models (41–43). Although many synthetic glycopeptides have been described for immunization purposes, only one group has reported the synthesis of glycopeptides containing an universal T-helper cell epitope so far. Indeed, carbohydrate–PADRE conjugates have been shown to raise high titers of IgG antibodies specific for lacto-*N*-fucopentaose II and for a dodecasaccharide derived from *S. typhimurium* (26). However, the immunogenicity of this synthetic compound was evaluated only in conventional C57BL/6 mice and the efficacy of the vaccine construct was not evaluated in a HLA restricted system.

In summary, PADRE-based glycoconjugates can address the limitations of the genetic restriction in humans by binding of the mouse serum antibodies to human Jurkat T-lymphoma cells that express the Tn antigen (30). As shown in Fig. 4, a positive binding of antibodies from mice vaccinated with Tn6-PAD glycopeptide was detected whereas no binding was observed when sera from mice immunized with the PAD control peptide were used.

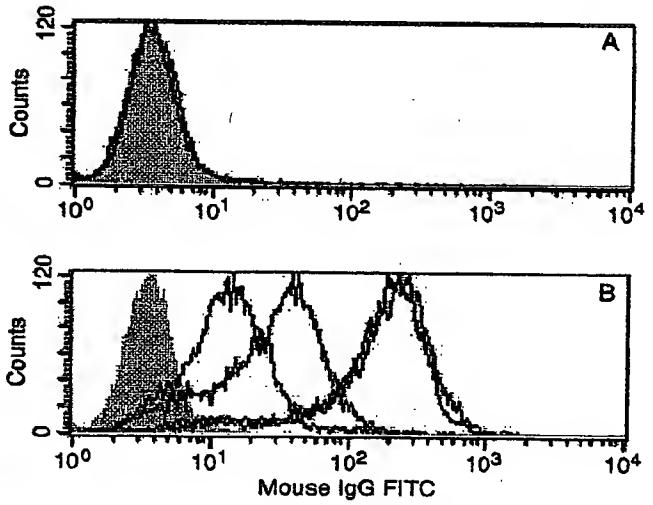


*Figure 4*. Native Tn recognition on tumor cells by Tn-PAD induced IgG antibodies. Sera (diluted 50 folds) from PAD (A) and Tn6-PAD (B) immunized mice were tested individually for the recognition of naive Tn on Jurkat cells by flow cytometry (revealed with antimouse IgG FITC antibodies, staining by secondary reagents alone (gray histograms) and by sera (thin line)). Results are shown for sera collected in HLA-DR4 Tg mice. Similar results were obtained with sera from HLA-DR1 Tg mice (not shown).

providing an effective T-cell help for carbohydrate-specific immune response based on an universal T-helper cell epitope. Such synthetic immunogens are particularly attractive for both their purity and accurate chemical definition which are essential features for safe clinical vaccines. Previous results showed that, although simple monovalent glycopeptides are efficient immunogens, the dendrimeric MAG system can induce an even stronger immune response (19). Moreover, using immunogenic peptides, multivalent constructs bearing 'universal' T-cell epitopes' have already been shown to be potent immunogens (44,45). The use of carbohydrate–PADRE glycoconjugates may thus be extended to the MAG system in order to further improve its immunogenicity and to provide an efficient approach for the development of anticancer vaccines with a broad human population coverage.

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# Synthesis and immunological evaluation of an antitumor neoglycopeptide vaccine bearing a novel homoserine Tn antigen

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**Abstract**—As part of our program on Tn-specific anti-tumor immunotherapy, our aim was to vary the nature of the aglyconic part of the tumor-associated Tn antigen (α-D-GalNAc-Ser/Thr). This report describes the synthesis of Fmoc-hSer-(α-D-GalNAc)-OH (4) in 19% overall yield from protected aspartic acid. The building block 4 was incorporated as trimeric clusters into a glycopeptide vaccine [MAG:Tn(hSer)3-PV], using solid-phase peptide synthesis. When injected in mice, the resulting MAG induces a strong antibody response, which recognizes native tumor-associated antigens (TAA) at the surface of human tumor cells. This approach may be extended to the use of other nonnatural TAA in order to improve half-life of synthetic anti-cancer vaccines.


Over the last years, numerous carbohydrate tumor-associated antigens (TAA) have been identified and used as potential targets for anti-cancer therapy: Tn, T, sialyl-Tn, Lewis antigens, and glycolipid-derived antigens (KH-1, globo H, GM2, ...).[1] To generate an efficient immune response, these TAA are usually linked to an immunogenic carrier protein.

To overcome the drawbacks associated with the protein conjugates (uncertainty in both composition and structure, low hapten density, irrelevant antibody production), we designed an efficient synthetic immunogen, the multiple antigen glycopeptide (MAG).[2,4] We prepared MAG vaccines displaying the Tn antigen (α-D-GalNAc-Ser/Thr) as the carbohydrate TAA. This antigen is overexpressed on epithelial tumors and is associated with many cancers including breast, prostate, lung, and pancreatic cancers.[5,6] To mimic the clustered motif encountered in vivo, we chose a tri-Tn mucin-like glycotope recognized by the tumor specific MLS-128 monoclonal antibody:[7] Ser(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr(α-D-GalNAc). The resulting MAG vaccine MAG:Tn3-PV, based on a dendrimeric lysine core carrying four copies of a $CD4^+$ T-cell peptide epitope together with the tri-Tn glycotope, is highly immunogenic in mice and afforded good protection in prophylactic and therapeutic vaccinations against the development of Tn-expressing tumor cells.[4] The anti-Tn antibody response is abolished when mice were depleted of CD4 T cells in vivo, showing the absolute requirement of the PV specific CD4 T-cell response.[4]

Preliminary results of our laboratory show that the amino-acid carrying the Tn antigen (Ser or Thr residues) contributes to the antibody recognition. Indeed, by varying the tri-Tn glycotope structure in the MAG, we obtained different monoclonal antibodies specific for the Ser(α-D-GalNAc)-Ser(α-D-GalNAc)-Ser(α-D-GalNAc)

*Abbreviations*: AAA, amino acid analysis; biot, biotine; DMF, dimethylformamide; ELISA, enzyme-linked immunosorbent assay; ESMS, electrospray mass spectrometry; FACS, fluorescent activated cell sorter; hSer, homoserine; MAP, multiple antigenic peptide; MAG, multiple antigenic glycopeptide; PV, poliovirus; RP-HPLC, reverse-phase high-performance liquid chromatography; Ser, serine; SPPS, solid-phase peptide synthesis; TAA, tumor-associated antigen.

*Keywords*: MAG: multiple antigenic glycopeptide; Tn antigen; Homoserine; Immunogenicity; Antibodies.

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sequence or the Ser(α-D-GalNAc)-Thr(α-D-GalNAc)-Thr(α-D-GalNAc) sequence (R. Lo Man and coll., unpublished results).

Approaches involving Tn antigen analogues have only been reported by a few laboratories: a neoglycopeptide with an analogue bearing a *C*-glycosyl linkage[8] and a protein glycoconjugate bearing a longer *O*-aliphatic aglycone.[9] In both cases the Tn analogue was incorporated as a monomer. Interestingly, the later vaccine construct was more antigenic than the natural Tn-based conjugate.[10]

In order to vary the nature of the aglyconic part of the Tn structure, we used the homo-serine (hSer) as the amino-acid carrier for the GalNAc residue. hSer allows to increase the distance of the carbohydrate residues from the peptide chain by one carbon atom as compared to its serine analog. This nonnatural amino-acid has already been included in a linear glycopeptide bearing a single tumor-associated disaccharide,[11] but, to our knowledge, the immunogenicity of this construct was not analyzed.

In the present paper, we prepared a new MAG bearing several α-D-GalNAc residues displayed as trimeric clusters on a hSer-hSer-hSer motif [MAG:Tn(hSer)3-PV] and we show that the injection of this neoglycopeptide in mice raises antibodies, which are able to recognize native TAA at the surface of human tumor cells.

*Preparation of glycosylated building block Fmoc-hSer-(α-D-GalNac)-OH 4 (Scheme 1):* For the synthesis of *N*-α-Fmoc-L-homoserine-α-*t*-butyl ester (Fmoc-hSer-O*t*Bu 1), commercially available *N*-α-Fmoc-L-aspartic acid-α-*t*-butyl ester was reduced to the corresponding alcohol.[12] This method avoids the formation of the Fmoc-γ-lactone occurring during the Fmoc-protection of hSer[11] and allows to obtain in a single step the Fmoc-hSer-O*t*Bu 1 (65%) appropriately protected for the glycosylation reaction.

The 3,4,6-tri-*O*-acetyl-2-azido-2-deoxy-β-D-galactopyranosyl chloride[13] was prepared in three steps starting from the tri-*O*-acetyl-D-galactal.[14] It was then added to the Fmoc-hSer-O*t*Bu 1 for the Koenigs–Knorr condensation.[15] Although trimethylsilyl trifluoromethane sulfonate (AgOTf)[16] was found to be the best catalyst for the condensation of serine derivatives, in our hands, the catalysts silver carbonate ($Ag_2CO_3$)/silver perchlorate ($AgClO_4$)[17] afforded the α-anomer in a better yield. Separation of the two anomers by flash chromatography was achieved after reduction and acetylation of the azido group (overall yield 88% based on **1**, α/β: 76/24).

The final deprotection of the *t*-butyl ester and the *O*-acetyl groups of **3**[17] afforded the glycosylated hSer building block **4** appropriately protected for the peptide synthesis with 19% overall yield.[18]

*Solid-phase synthesis of MAG:Tn(hSer)3-PV (Fig. 1):* MAG:Tn(hSer)3-PV was assembled by the conventional solid-phase peptide methodology (Wang resin) using Fmoc chemistry as described previously.[2,4] The protected amino acids were incorporated manually into the peptide sequence using 2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (TBTU)/1-hydroxybenzotriazole (HOBT)/diisopropylethylamine (DIEA) as the coupling reagent.[19,20] Fmoc protection was removed with 20% piperidine in DMF.

Three glycosylated building blocks [Fmoc-hSer(α-D-GalNAc)-OH 4] were incorporated successively as their pentafluorophenyl (Pfp) esters in the presence of HOBT.[21] The Pfp esters were prepared by addition of

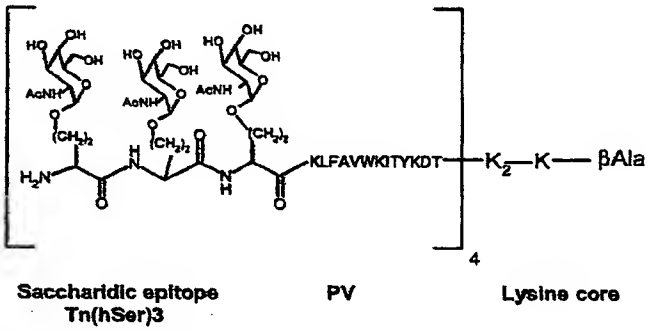
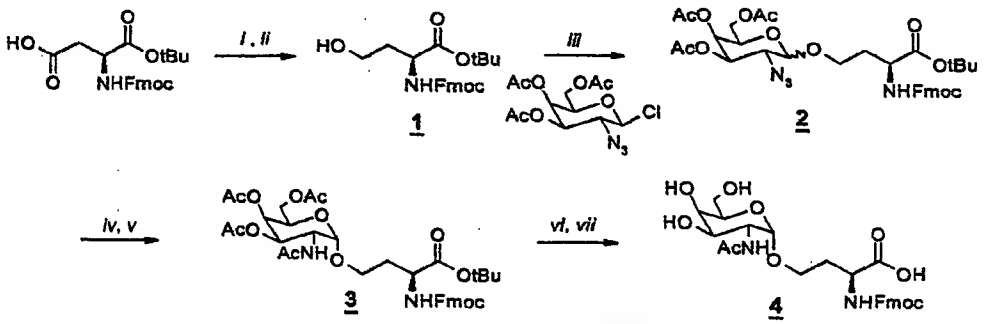



Figure 1. Schematic representation of MAG:Tn(hSer)3-PV.




Scheme 1. Preparation of glycosylated homoserine (Fmoc-hSer-(α-D-GalNac)-OH). Reagents, conditions, and yields: (i) EtOCOCl, $Et_3N$, THF, 1 h, −10 °C; (ii) $NaBH_4$, $H_2O$, 65% (two steps); (iii) $Ag_2CO_3$, $AgClO_4$, $CH_2Cl_2$, toluene; (iv) $NiCl_2$, $H_3BO_3$, $NaBH_4$, EtOH; (v) $Ac_2O$, 67% (three steps); (vi) HCOOH, 98%; (vii) MeONa, MeOH, 46%.

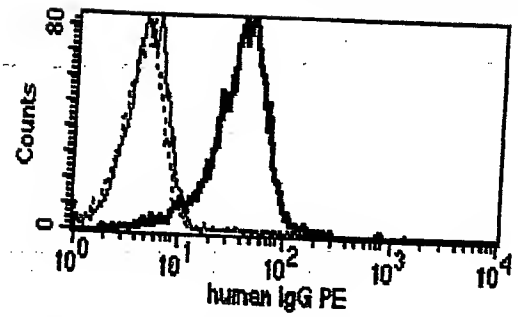



Figure 2. Recognition of a human tumor cell line by sera from MAG:Tn(hSer)3-PV-primed mice. Mice were injected on days 0 and 21 with MAG:Tn(hSer)3-PV (bold line) or with MAP:PV devoided of Tn (thin line) in alum, and sera collected on day 28 were analyzed by FACS for recognition of Tn-positive Jurkat cells. Dotted line corresponds to unstained cells.

1,3-diisopropylcarbodiimide to the glycosylated amino acid and pentafluorophenol in dry dichloromethane;[22] they were used directly after concentration without purification. The products were cleaved from the resin with aqueous trifluoroacetic acid (TFA), triisopropylsilane, $H_2O$, phenol.

The MAG (Fig. 1) was purified by reverse-phase high-performance liquid chromatography with gradient performed with water (0.1% TFA)/acetonitrile on a C18 column and it was characterized by amino acid analysis (AAA) and electrospray mass spectrometry (ESMS).[23]

*In mice, MAG:Tn(hSer)3-PV induces specific antibodies which recognize a human tumor cell line (Fig. 2):* We analyzed the immunogenicity of MAG:Tn(hSer)3-PV in BALB/c mice. Sera from immunized mice were tested by enzyme-linked immunosorbent assay (ELISA) against a biotinylated synthetic Tn(hSer) cluster [Tn(hSer)3-G6K(biot)G] coated on streptavidin plates.[24] The non-glycosylated analogue was used as a control for background reactivity. Immunization with the MAG induced specific IgG antibodies, which recognized the Tn(hSer)3 cluster (data not shown).

In a previous study, we demonstrated that anti-Tn IgG antibody titers obtained by immunization with a linear glycopeptide bearing three D-serine residues were similar to those obtained with a glycopeptide containing three L-serine, as measured by FACS using human tumor cells.[25] Indeed this method gives a more accurate view of the 'natural' display of TAA at the cell surface for antibody recognition.

To ensure that the antibodies induced by the MAG:Tn(hSer)3-PV were able to recognize the native Tn antigen on tumor cells, we analyzed the binding of mouse sera to human Jurkat T-lymphoma cells that express the Tn antigen. Figure 2 shows that these cells were recognized by the MAG-induced antibodies, showing the biological relevancy of the immune response.

In conclusion, we describe (i) an efficient route for the synthesis of a glycosylated homoserine building block as a Tn antigen analogue and (ii) its incorporation in an anti-tumor vaccine neoglycopeptide by SPPS. We also show that the immune response induced in mice by this vaccine is biologically relevant since the resulting antibodies recognize a human tumor cell line.

These results open new perspectives for the rational design of synthetic anti-cancer vaccines with longer half-life. Indeed the incorporation of nonnatural Tn analogues in glycopeptide vaccines should prevent the in vivo degradation of the natural glycosyl-serine linkage.

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(m, 1H, CH hSer), 3.97 (m, 1H, H-2), 3.66 (br d, 1H, H-4), 3.55 (m, H-3, H-5), 3.44, 3.40 (m, H-6, H-6'), 3.55, 3.24 ($CH_2O$ hSer), 1.94 (m, 1H, $CH_2CH$), 1.77 (m, 4H, $CH_2CH$, $CH_3$ Ac) ; $^{13}C$ NMR ($(CD_3)_2SO$, 100 MHz): $\delta$ 174.76, 170.64 (CONH, COOH), 156.92 (OCONH), 144.72, 144.64, 141.57, 141.55 (C Phe), 128.50, 127.95, 126.11, 126.06, 120.96 (CH Phe), 98.50 (C-1), 72.14, 68.96, 68.73 (C-3, C-4, C-5), 66.48 ($CH_2$ Fmoc), 64.65 ($CH_2O$), 61.44 (C-6), 52.00 (CH hSer), 50.60 (C-2), 47.52 (CH Fmoc), 31.79 ($CH_2CH$), 23.54 ($CH_3$ Ac). FABMS for $C_{27}H_{32}N_2O_{10}$: (calcd 544.21) $m/z$ 567.3 $[M+Na]^+$.


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# A Fully Synthetic Therapeutic Vaccine Candidate Targeting Carcinoma-Associated Tn Carbohydrate Antigen Induces Tumor-Specific Antibodies in Nonhuman Primates

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## ABSTRACT

**We recently developed an efficient strategy based on a fully synthetic dendrimeric carbohydrate display (multiple antigenic glycopeptide; MAG) to induce anticarbohydrate antibody responses for therapeutic vaccination against cancer. Here, we show the superior efficacy of the MAG strategy over the traditional keyhole limpet hemocyanin glycoconjugate to elicit an anticarbohydrate IgG response against the tumor-associated Tn antigen. We highlight the influence of the aglyconic carrier elements of such a tumor antigen for their recognition by the immune system. Finally, we additionally developed the MAG system by introducing promiscuous HLA-restricted T-helper epitopes and performed its immunological evaluation in nonhuman primates. MAG:Tn vaccines induced in all of the animals strong tumor-specific anti-Tn antibodies that can mediate antibody-dependent cell cytotoxicity against human tumor. Therefore, the preclinical evaluation of the MAG:Tn vaccine demonstrates that it represents a safe and highly promising immunotherapeutic molecularly defined tool for targeting breast, colon, and prostate cancers that express the carbohydrate Tn antigen.**

## INTRODUCTION

Cancerous transformation is very often associated with a dysregulation of the glycosylation processes leading to altered carbohydrate patterns at the surface of cancer cells (1). This results in the expression of various carbohydrate antigens such as blood group-related Tn, T, sialyl-Tn, sialyl-T antigens (family of T antigens) associated with carcinomas (2) or glycolipidic GM2, GD2, and GD3 associated with melanomas (3). Some of these tumor-associated carbohydrate antigens, involved in metastatic processes and associated with a poor prognosis, represent an excellent target for immune intervention after tumor resection and chemotherapy treatments to avoid cancer recurrence (4).

In a large variety of epithelial cancers such as breast, ovarian, colorectal, pancreatic, or prostate cancers, highly *O*-glycosylated mucins are strongly affected in their carbohydrate patterns displaying nonglycosylated as well as abortive glycosylated products such as Tn, T, sialyl-Tn, and sialyl-T antigens (2, 5–8). Naked peptides derived from variable number tandem repeat of MUC1 sequences have been used for the design of immunotherapeutic vaccine to elicit cellular and humoral antitumoral immune responses (9–11). However, some MUC1 peptide sequences showed T-cell immunosuppressive activity (12). Moreover, anti-MUC1 natural antibodies found in cancer patients with a favorable prognosis preferentially recognize glycosylated forms of MUC1 peptides (13). Therefore, the family of T antigens represents a suitable candidate for immune intervention. Moreover, these truncated glycosylation products are expressed in fetal life and remain mainly cryptic in normal adult tissues, thus limiting risks of autoimmunity.

Up to now, to elicit B-cell responses specific for these carbohydrate tumor antigens, they have been chemically linked to a carrier protein, such as keyhole limpet hemocyanin (KLH), to provide T-cell help required for antibody production. Several gangliosides-KLH glycoconjugates have reached Phase II/III clinical trials (14). For the family of T antigens, the administration of sialyl-Tn-KLH glycoconjugate in DETOX adjuvant was correlated with an increased survival in patients with metastatic breast cancers (15). In contrast to the large ganglioside structures, short haptenic Tn, T, sialyl-Tn molecules require to be associated as cluster of at least two to three units to mimic native forms found on mucins (16–18), whereas a single unit does so poorly (19). This requirement for repetitive carbohydrate units reflects the composition of the mucin substrate, highly enriched in consecutive serine or threonine residues, but also the *O*-glycosylation process occurring in cancer cells. For instance, the *N*-acetylgalactosaminyltransferase T3, responsible for the glycosylation of consecutive threonine residues, is overexpressed in adenocarcinomas leading to the expression of Tn clusters (20). Therefore, the strategy of carbohydrate clustering has greatly improved the immunogenicity of these short haptenic molecules allowing the recognition of native carbohydrate structures on tumor cells (21–23).

On protein glycoconjugates, the carbohydrate density that can be achieved is highly variable. Advances in the design of appropriate linkers and in conjugation procedures have improved the efficiency of chemical coupling to obtain elevated cabohydrate:carrier protein ratio (24). However, the use of a limited number of carrier proteins to conjugate carbohydrate antigens may limit the efficacy of these glycoconjugates. Indeed, the immune response to the carrier is much more superior to the one directed against the carbohydrate antigens, and this may lead to carrier-induced epitopic suppression (25, 26). In addition, an accurate molecular definition of glycoconjugates in terms of composition and structure can only be achieved by the full chemical synthesis of immunogens.

Following an entirely chemical synthesis process, we developed dendrimeric MAG as an alternative strategy to glycoprotein conjugates. MAG is based on the linking of a high density of carbohydrates to a nonimmunogenic lysine core to focus the immune response to the haptenic moiety (27, 28). We applied the MAG strategy to the Tn antigen (α-GalNAc-Ser/Thr) with a trimeric form, which was associated with a T-helper peptide to allow the induction of a T cell-dependent IgG antibody response against the Tn tumor antigen. When administered with alum, in either therapeutic or prophylactic protocols, these MAG conjugates increased up to 80% the survival of tumor-bearing mice (23, 28). In previous attempts, fully synthetic lipoglycopeptides with a dimeric Tn associated with a palmitoyl core induced IgM but failed to elicit IgG antibodies required for antibody-dependent cellular cytotoxicity (ADCC) against cancer cells (29). The same strategy developed with the carcinoma-associated Lewis$^y$ antigen also resulted in the sole IgM antibody production (30). In many

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cases, KLH glycoconjugates also induced IgM antibodies but no or moderate IgG in mice and humans (4). This major issue can be partially overcome by using a strong adjuvant such as QS21. In contrast, the multiple antigenic glycopeptide (MAG) showed its immunological potency and therapeutic effects in mice using the harmless and commonly used vaccine adjuvant, aluminum hydroxide.

In the present study, to optimize the development of MAG:Tn immunogens, we first investigated the influence of the amino acid carrying the GalNAc to design the most appropriate antigenic and immunogenic Tn cluster. The efficacy of the MAG strategy was then tested by comparing the MAG:Tn with its KLH-Tn counterpart in inducing anti-Tn IgG antibodies in mice. Finally, to apply the MAG:Tn for human vaccination we designed two MAG:Tn compounds in which "universal" $CD4^+$ T-cell epitopes known to stimulate effective T-helper cell responses in human populations with many HLA diversity were introduced. These MAG were tested in nonhuman primates (macaques and green monkeys) and found to induce strong anti-Tn IgG antibodies capable of specifically recognizing Tn-expressing human tumor cells. Moreover, these antibodies were able to mediate ADCC against Tn-positive human tumor cells.

## MATERIALS AND METHODS

**Chemical Syntheses of Linear and Dendrimeric Glycopeptides.** The MUC glycopeptides (SLSYTNPAV and ALGSTTPPA series) were synthesized on an Applied Biosystems Pioneer peptide synthesizer using continuous-flow Fmoc chemistry and 2-(1H-9-azabenzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate/diisopropylethylamine as the coupling reagents. The protected glycosylated building blocks [Fmoc-Ser(α-GalNAc(OAc)$_3$)-OH or Fmoc-Thr(α-GalNAc(OAc)$_3$))-OH] were incorporated manually using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole activation. The synthesis of the other peptides and glycopeptides was performed as described previously by solid-chemistry (23, 31, 32). Briefly, the protected amino acids were incorporated manually into the peptide sequence using 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate/1-hydroxybenzotriazole/diisopropylethylamine as the coupling reagents. Fmoc protection was removed with 20% piperidine in dimethylformamide. The glycosylated building blocks [Fmoc-Ser(α-GalNAc)-OH, Fmoc-Thr(α-GalNAc)-OH, or Fmoc-hSer(α-GalNAc)-OH (32)] were incorporated as their crude pentafluorophenyl esters in the presence of 1-hydroxybenzotriazole. The products were cleaved from the resin with aqueous trifluoroacetic acid, triisopropylsilane, $H_2O$, and phenol. When necessary, deacetylation of the sugar residue was achieved with a catalytic amount of sodium methoxide in methanol at pH 11. Peptides and glycopeptides were purified by reverse-phase high-performance liquid chromatography using a Perkin-Elmer pump system with a UV detector at 230 nm. The column was a Waters Delta Pak C18 (15 μ, 300Å, 7.8 × 300 mm), and the gradient was performed with water (0.1% trifluoroacetic acid)/acetonitrile over 20 min. The compounds were characterized by amino acid analysis and mass spectrometry. Mass spectra were recorded by electrospray (electrospray mass spectrometry) in the positive mode on a Quattro-LCZ or LCT of mass spectrometer (Micromass, Manchester, United Kingdom). The sample was dissolved at 10 mM concentration in water:acetonitrile (1:1) with 0.1% formic acid. Amino acids and electrospray mass spectrometry mass analyses were conformed to expected products.

**Immunization of Mice.** BALB/c mice (CER Janvier, Le Genest St Ile, France) were i.p. immunized with 1 or 10 μg of MAG:Tn3-PV, MAG:Tn(S)3-PV, MAG:Tn(hS)3-PV, or KLH-Tn(c) together with alum (Serva, Heidelberg, Germany) or QS21. KLH-Tn(c) and QS21 were from previous studies (4, 21). For comparative studies between MAG and KLH conjugates, mice received 0.34 μg and 1 μg of Tn cluster, respectively, per immunization. Immunostimulatory oligonucleotides containing unmethylated CpG motifs were synthesized by Proligo (Paris, France). CpG 1826 was used in mice. CpG 2006, active on human peripheral blood mononuclear cell, was used for primate immunization. The anti-CD40 mAb (FGK45) was prepared from ascitic fluids.

**Immunization of Primates.** The 9 African Green Monkeys, *Chlorecebus sabaeus*, used in this study were all simian immunodeficiency virus and simian T-lymphotropic virus negatives. Both male and female juveniles (under 3 years of age) and subadults (over 3–4 years) were included. Animals lived in Senegal (West Africa) and were caught from the wild using nets and baits. After a minimal period of 60 days of adaptation to captivity that included observation and veterinary carefulness, animals entered immunization experiments. Animal care operations were in compliance with the regulations detailed under the Guide for the Care and Use of Laboratory Animals. Immunizations were performed s.c. on the back region of monkeys (injections of 0.2 ml/animal/immunization) after an anesthesia with ketamine. Animals received three to four injections of 500 μg of MAG:Tn3-TT or control MAP:TT in the presence of 1 mg of aluminum hydroxide with or without 100 μg of ODN 2006. Animals were bled before and after each immunization (5–10 ml of blood/animal). On the day of withdrawals, monkeys were examined and weighed.

The 8 adults, *Macaca mulatta*, were imported from China and were housed at the animal house of Rennemoulin (France) in single cages in accordance with the European Community guidelines for animal care. Macaques were injected previously with noninfectious antigenic formulations containing simian immunodeficiency virus Tat and Nef proteins (# 250, 254, 327, and 340) or simian immunodeficiency virus nef only (#279, 328, 332 and 338) and were divided in two groups, respectively. In each group, 1 animal received adjuvant alone (alum plus ODN 2006) and 3 others received adjuvant (alum plus ODN 2006) together with 500 μg of MAG:Tn3-TT or MAG:Tn(S)3-PADRE. Immunizations were performed i.m. (4 injections of 0.5 ml/animal/immunization) after an anesthesia. Animals were bled before and at the time of immunization (10 ml of blood/animal). Blood samples were used to analyze sera for anti-Tn antibodies and proliferative response of peripheral blood lymphocyte to TT and PADRE peptides containing T-cell epitopes.

No adverse reaction or local inflammation was noted at the sites of injection, and the weight of the animals did not vary by >10% during the study. By the end of the immunization procedure, all of the animals were healthy and increased in weight.

**Antibody Detection by ELISA and by Fluorescence-Activated Cell Sorting.** Sera were tested as described previously (28) for anti-Tn antibodies by ELISA using biotinylated synthetic Tn cluster glycopeptides (see Table 1) coated on streptavidin plates. aOSM (kindly provided by Dr. Eduardo Osinaga, Facultad de Medicina, Montevideo, Uruguay) and KLH were directly coated on plates. Goat antimouse IgG or goat antihuman IgG peroxidase conjugate (Sigma, St. Louis, MO) was used. Sera from mice and primates were tested by flow cytometry on Tn-expressing human tumor cell lines, Jurkat and MCF-7, and on Tn-negative tumor cells, T2 and MDA231. Binding of antibodies to the cells was revealed with goat antimouse IgG antibody conjugated to FITC or goat antihuman IgG antibody conjugated to phycoerythrin and paraformaldehyde-fixed cells were analyzed on a fluorescence-activated cell sorter. Statistical analysis was performed by a permutation test using the StatXact software (Cytel Software Corporation, Cambridge, MA).

**Competition Assay with Glycosylated MUC1 Peptides.** Sera from green monkeys were tested for recognition of MUC1 peptide sequence in liquid phase using a competition assay. For this purpose, Tn3-G6KG-biot was plated on streptavidin-coated plates and then incubated for 15 min with sera together with serial concentration of nonbiotinylated Tn3-G6KG or MUC1 glycopeptides (competitors). After washing, IgG bound to Tn3-G6KG-biot/streptavidin plates was detected as described above. After this procedure, the concentration of nonbiotinylated Tn3-G6KG and MUC1 glycopeptides giving $IC_{50}$ of the signal obtained with the serum alone were determined. For each experiment, $IC_{50}$ for all of the MUC1 glycopeptides were normalized with the $IC_{50}$ obtained with the nonbiotinylated Tn3-G6KG in the same experiment. Results are expressed as $IC_{50}$ (MUC1 glycopeptide)/$IC_{50}$ (nonbiotinylated Tn3-G6KG) for each serum and as a mean of value obtained in two to three experiments.

**ADCC Assay.** Sera from primates were tested by a $^{51}Cr$ release assay for their capacity to mediate ADCC of tumor cells performed by a human natural killer (NK) cell clone (kindly given by Dr. Fathia Mami-Chouaib, IGR, Villejuif, France) as effector cell. For the cytotoxic assay, tumor-target cells were labeled with $^{51}Cr$, then incubated with serum for 20 min. at 4°C, washed twice, and plated at $10^4$ cells/well. NK cell clone cells were added for 4 h at various E:T ratios. The percentage of specific lysis was calculated as 100 × (experimental release − spontaneous release)/(maximal release − spontaneous release).

Table 1 *List of compounds used in this study*

| Compound[a,b,c] | Peptidic backbone | | Glycosidic moiety | | | | |
|---|---|---|---|---|---|---|---|
| | Structure | Sequence[b] | Tn structure | Tn copies | Use | Host | Reference |
| MAG:Tn3 | Dendrimer | - | S*T*T* | 12 | Vacc. | Mouse | This study |
| MAG:Tn3-PV | Dendrimer | PV | S*T*T* | 12 | Vacc. | Mouse | (1) |
| MAG:Tn(S)3-PV | Dendrimer | PV | S*S*S* | 12 | Vacc. | Mouse | This study |
| MAG:Tn(hS)3-PV | Dendrimer | PV | hS*hS*hS* | 12 | Vacc. | Mouse | (2) |
| MAP-TT | Dendrimer | TT | - | 0 | Vacc. | Primate | This study |
| MAG:Tn3-TT | Dendrimer | TT | S*T*T* | 12 | Vacc. | Primate | This study |
| MAG:Tn(S)3-PADRE | Dendrimer | PADRE | S*S*S* | 12 | Vacc. | Primate | This study |
| KLH-Tn(c) | Linear/protein | KLH | cluster | 951 | Vacc. | Mouse | (3) |
| Tn3-G6K(biot)G | Linear | Poly-gly | S*T*T* | 3 | ELISA | - | (4) |
| Tn(S)3-G6K(biot)G | Linear | Poly-gly | S*S*S* | 3 | ELISA | - | (5) |
| Tn(hS)3-G6K(biot)G | Linear | Poly-gly | hS*hS*hS* | 3 | ELISA | - | This study |
| Tn(T)3-G6K(biot)G | Linear | Poly-gly | T*T*T* | 3 | ELISA | - | This study |
| SLS*YT*NPAV | Linear | MUC1 | S* and T* | 2 | ELISA | - | This study |
| ALGS*TT*PPA | Linear | MUC1 | S* and T* | 2 | ELISA | - | This study |
| ALGS*T*TPPA | Linear | MUC1 | S*T* | 2 | ELISA | - | This study |
| ALGS*T*T*PPA | Linear | MUC1 | S*T*T* | 3 | ELISA | - | This study |
| S*T*APPAHGV | Linear | MUC1 | S*T* | 1 | ELISA | - | This study |

[a] MAP and MAG refer to dendrimeric compounds based on the $(Lys)_2$-Lys-β-Ala core; Tn3-G6K(biot)G, Tn(S)3-G6K(biot)G, Tn(T)3-G6K(biot)G, and Tn(hS)3-G6K(biot)G were abbreviated for simplicity as Tn3, Tn(S)3, Tn(T)3, and Tn(hS)3, respectively.

[b] T-helper peptide sequences: PV = KLFAVWKITYKDT is restricted by H-$2^d$; TT = QYIKANSKFIGITEL is restricted by HLA-DR1, DR3, DR5, DR7, and DRw52; PADRE = AKXVAAWTLKAAA (X = cyclohexylalanine) is restricted by HLA-DR1, DR4w4, DR4w14, DR5, and DR2w2a.

[c] *, α-DGalNAc; biot, biotine; hS, homoserine.

## RESULTS

**Influence of the Aglyconic Backbone on the Tn Glycotope Recognition.** Haptenic Tn, T, and sialyl-Tn molecules require to be associated as clusters of at least two to three units to mimic native forms found on mucins (16–18). However, thus far the influence of amino acids (Ser or Thr) carrying the α-D-GalNAc within the cluster has never been considered. Therefore, we first investigated the role of the Tn aglyconic backbone for antibody recognition. We immunized mice with three different MAG, MAG:Tn3-PV, MAG:Tn(S)3-PV, and MAG:Tn(hS)3-PV (Table 1), and we analyzed immune sera for the recognition of their homologous synthetic Tn cluster, Tn3, Tn(S)3, and Tn(hS)3. In these conditions, high anti-Tn IgG antibody titers were found in all groups of mice (Fig. 1*A*). When sera induced by MAG:Tn3-PV were analyzed for the recognition of different heterologous Tn clusters (Table 1), these antibodies also recognized Tn(S)3 and Tn(T)3 motifs but not the non-natural Tn(hS)3 cluster. The same was true for antibodies induced by the MAG:Tn(S)3-PV. In contrast, antibodies induced by MAG:Tn(hS)3-PV recognized efficiently the synthetic Tn(hS)3 cluster, but poorly Tn3, and not Tn(S)3 and Tn(T)3, demonstrating a highly reduced cross-recognition of the GalNAc moiety for non-natural amino acids. In addition to these data, we found that among the Tn-specific monoclonal IgG antibodies we produced, some recognized the synthetic Tn(S)3 but not

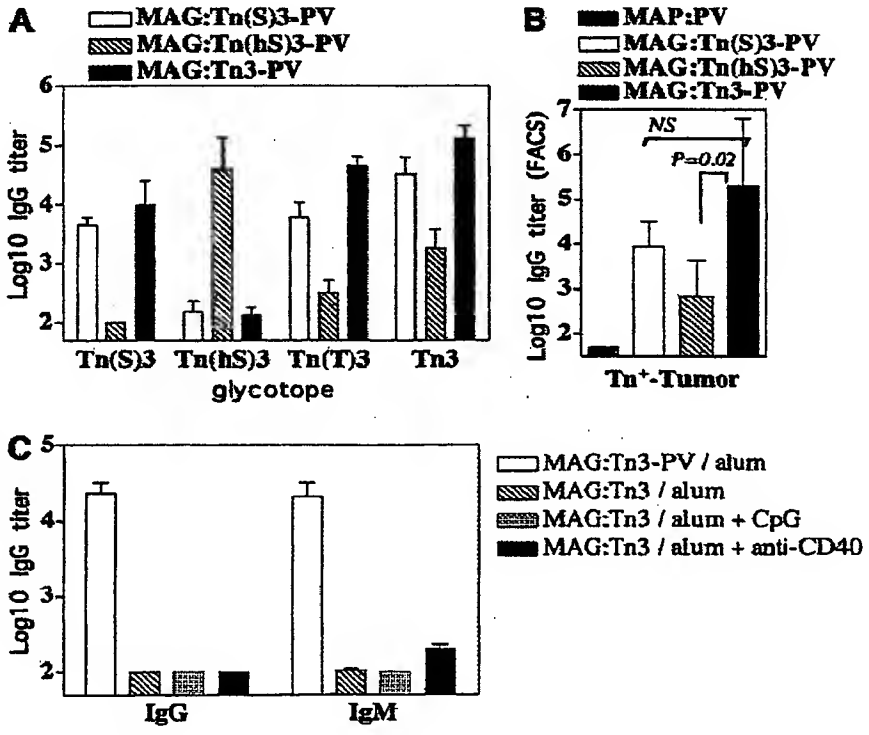


Fig. 1. Influence of the glycosidic display on the generation of antibodies that recognize the native form of Tn. *A*, mice ($n = 5$) were immunized three times with 10 μg of multiple antigenic glycopeptide (*MAG*):Tn(S)3-PV, MAG:Tn(hS)3-PV, and MAG:Tn3-PV in alum, and sera collected after the last boost were tested for anti-Tn IgG antibodies by ELISA using synthetic Tn(S)3, Tn(hS)3, Tn(T)3, or Tn3 as indicated. *B*, the same sera were tested for native Tn recognition by fluorescence-activated cell sorter using Tn-positive human tumor cells (Jurkat). Statistical analysis is indicated by the *P* or by *NS* for nonsignificant statistical difference. *C*, mice ($n = 4$) were immunized twice, on days 0 and 21, with 10 μg of MAG:Tn3-PV or MAG:Tn3 in alum alone or together with 50 μg of CpG or with 3 × 100 μg of anti-CD40 monoclonal antibody. Sera collected at day 28 were analyzed for anti-Tn IgG and IgM antibodies by ELISA using synthetic Tn3; *bars*, ±SD.

other Tn clusters based on a different backbone.[6] To additionally evaluate this phenomenon, we compared the capacity of the antibodies induced by the different MAG immunogens to recognize the "native form of Tn" on tumor cells by flow cytometry (Fig. 1*B*). No significant difference was found for the recognition of the Tn-positive Jurkat cell by antibodies elicited by MAG:Tn3-PV or with MAG:Tn(S)3-PV, whereas MAG:Tn(hS)3-PV induced antibodies displayed low reactivity for native Tn on Jurkat (Fig. 1*B*). Altogether, these results show that the Tn antigen/antibody interaction does not involve the sole carbohydrate moiety even when it is displayed as a cluster. However, as long as immunogens are built by *O*-glycosylation of natural serine and threonine, there exists sufficient diversity of the polyclonal IgG response to accommodate the large variability of mucin sequences and to allow the efficient recognition of the native forms of the Tn antigen.

**T-Cell Help Requirement to Induce Anti-Tn Antibodies with MAG Glycoconjugate.** We showed previously that *in vivo* depletion of the CD4+ T-cell compartment abrogates the induction of anti-Tn antibodies and the protection afforded by the MAG:Tn3-PV against the growth of Tn-expressing tumor in mice (23). We sought to determine whether we could bypass the requirement of T-cell help to induce anticarbohydrate antibodies by directly activating B cells. Such direct activation could be achieved by the cross-linking of membrane immunoglobulin by repetitive carbohydrate units (such as those found in bacterial polysaccharides) and/or by toll-like receptor or CD40 triggering. For this purpose, a MAG:Tn3 containing four Tn3 clusters devoided of peptide T-cell sequence was synthesized (Table 1). As shown in Fig. 1*C*, the multimeric MAG:Tn3 administered in alum did not induce any anti-Tn antibody responses. Neither TLR9 triggering by coinjecting CpG nor an anti-CD40 monoclonal antibody treatment together with MAG:Tn3 immunization led to the induction of anti-Tn antibodies (Fig. 1*C*) demonstrating the absolute requirement of a CD4+ T-cell help to induce antibodies against the monosaccharidic Tn antigen.

**Comparison of KLH and MAG Glycoconjugates.** Several KLH glycoconjugates developed with tumor-associated carbohydrates have entered in Phase II/III clinical trials (4, 14). These glycoconjugates were more or less efficient in inducing IgG antibodies required for ADCC. Therefore, we next performed a comparative study in mice of the immunogenicity of MAG:Tn3-PV *versus* its KLH-Tn(c) counterpart using different adjuvant settings. In contrast to the MAG, when KLH-Tn(c) was administered in alum, strong anti-KLH antibodies were induced but no anti-Tn IgG as tested by ELISA (Fig. 2*A*). In both cases, IgM antibodies specific for Tn were induced (Fig. 2*B*). We next compared KLH and MAG glycoconjugates injected with a stronger adjuvant such as QS21 (Fig. 2*A*). In these conditions, both KLH and MAG Tn-conjugates elicited anti-Tn IgG antibodies regardless of the synthetic Tn cluster used for detection. The nonglycosylated backbone was not recognized by any of the mouse sera (data not shown). Anti-Tn titers were significantly higher in the case of MAG and were maintained for >3 months after the last boost, indicating an induction of long-lived plasma cells (Fig. 2*C*).

When we tested sera from KLH-Tn(c) and MAG:Tn3-PV in QS21 immunized mice for Jurkat cells recognition, again the antibody titers induced by the MAG compound were significantly superior to those induced by the KLH glycoconjugate (Fig. 2*D*). However, no significant difference was observed when the Tn-positive MCF7 cells were used as a source of native Tn antigen. KLH naturally displays the glycosidic T antigen [Gal(β1–3)-GalNAc; Ref. 33] and, therefore, the reactivity of sera from animals immunized with KLH-Tn(c) most likely reflects the recognition of both Tn and T antigens on MCF7, whereas MAG:Tn3-PV induced antibodies are specifically directed against Tn. In contrast, Jurkat cells do not display the T antigen at the cell membrane (34) due to a lack of β1–3 galactosyl-transferase expression (35).

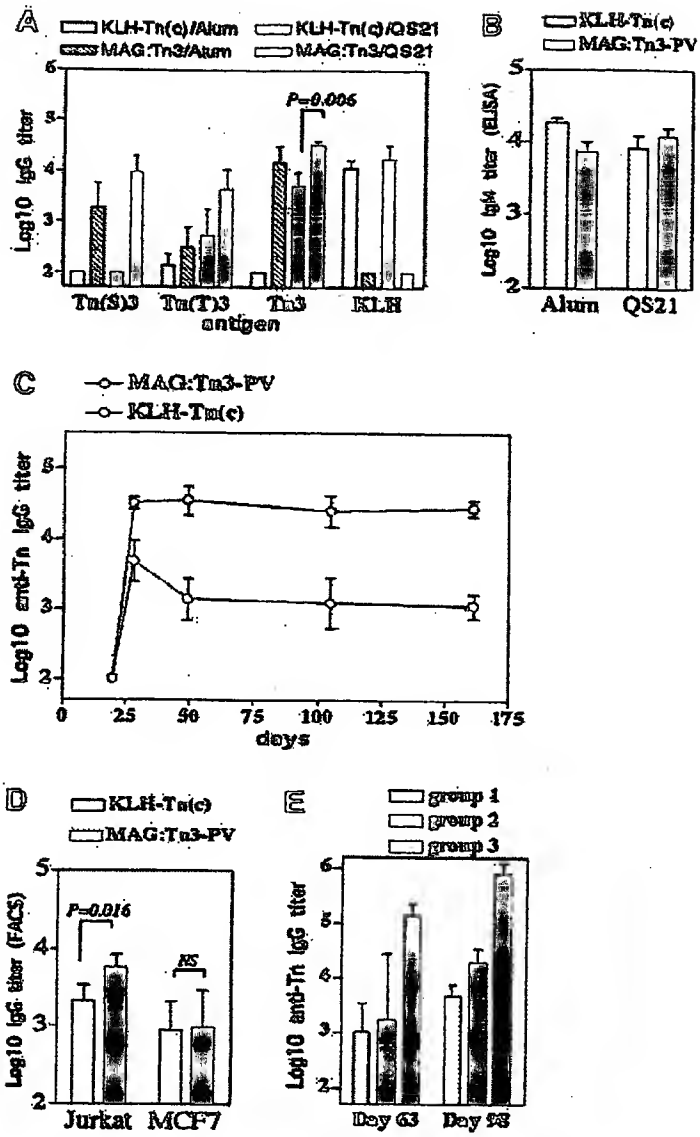


Fig. 2. Superior efficacy of multiple antigenic glycopeptide (*MAG*) over keyhole limpet hemocyanin (*KLH*) glycoconjugate in its ability to induce anti-Tn IgG antibodies. (*A–D*) BALB/c mice were immunized on days 0, 21, and 42 with KLH-Tn(c; 15.6 μg, *i.e.*, 1 μg of Tn cluster) or MAG:Tn3-PV (1 μg, *i.e.*, 0.34 μg of Tn cluster) mixed with alum (*A* and *B*) or QS21 (*A–D*). *A* and *B*, sera were collected 1 week after the second (QS21 group; $n = 9$) or the third (alum group, $n = 5$) immunization and tested by ELISA as indicated for anti-Tn3 or anti-KLH IgG antibodies (*A*) or anti-Tn3 IgM (*B*). *C*, sera from mice ($n = 9$) immunized with indicated glycoconjugate in QS21 and collected at different time points were tested for anti-Tn antibodies using synthetic Tn3. *D*, sera from mice immunized with glycoconjugates in QS21 were tested for native Tn recognition by fluorescence-activated cell sorter using the Tn-positive Jurkat and MCF7 tumors. *E*, on days 0, 7, 14, and 42, mice ($n = 3$) were immunized with 1 μg of KLH-Tn(c; groups 1 and 2) or 1 μg of MAG:Tn3-PV (group 3) mixed with QS21. On day 70, mice were recalled with KLH-Tn(c; group 1) or MAG:Tn3-PV (groups 2 and 3). Sera collected before (day 63) or after (day 98) the last recall were tested by ELISA for anti-Tn IgG against synthetic Tn3. Statistical analysis is indicated by the *P* or by *NS* for nonsignificant statistical difference; *bars*, ±SD.

Finally, we analyzed whether the B-cell response induced by the KLH glycoconjugate could be recalled and be additionally increased by the MAG. After three injections of KLH-Tn(c) in QS21, mice were recalled with MAG:Tn3-PV or KLH-Tn(c). In these conditions, no memory

[6] Unpublished observations.

response was recalled by the KLH or the MAG glycoconjugates boosting injection (Fig. 2*E*). In contrast, after the same schedule, MAG:Tn3-PV could additionally recall the anti-Tn IgG response induced by previous MAG:Tn3-PV immunizations. Altogether, these data clearly show the qualitative and quantitative superior efficacy of MAG:Tn3-PV over KLH-Tn(c) in inducing an anti-Tn immune response.

**Induction of Tn-Specific Antibodies by "Humanized" MAG in Nonhuman Primates.** We next designed MAG for human vaccination by introducing either a TT (36) or a PADRE (37) peptide that allows a broad coverage of HLA diversity (Table 1). We showed recently that linear Tn(S)6-PADRE induced anti-Tn antibodies in HLA-DR1 and -DR4 transgenic mice (38). Likewise, the new dendrimeric MAG, MAG:Tn(S)3-PADRE, and MAG:Tn3-TT (Table 1), also induced anti-Tn antibodies in these HLA transgenic mice (data not shown). Therefore, we next evaluated the potency of these MAG in two nonhuman primate species. In a first experiment, 2 groups of 3 macaques were immunized with MAG:Tn(S)3-PADRE and MAG: Tn3-TT with alum and CpG oligonucleotide as adjuvants. Control animals received the adjuvant preparation alone. IgG antibodies specific to Tn were detected by ELISA in all of the animals immunized with MAG but not in controls (Fig. 3, *A* and *B*). We also assessed the peripheral blood lymphocyte proliferative response to CD4+ T-cell peptides in vaccinated macaques after the last boost. TT-specific T-cell responses were detected in all of the animals vaccinated with MAG:Tn3-TT. PADRE-specific T-cell responses were found in 2 of 3 animals vaccinated with MAG:Tn(S)3-PADRE, although of lower intensity as compared with the TT responses (Fig. 3*C*).

Next, we carried an experiment with the MAG:Tn3-TT in green monkeys, to additionally evaluate the immunogenicity of this MAG when administered with alum alone or with CpG ODN. Control groups consisted of dendrimeric MAP:TT devoid of Tn residues. Anti-Tn IgG titers were already detectable after the first immunization (Fig. 3*D*). When green monkeys were immunized with MAG:Tn3-TT with alum as the sole adjuvant, 2 animals developed anti-Tn IgG after two immunizations and the third animal required a total of four injections to develop anti-Tn antibodies (Fig. 3*E*).

Finally, we tested the ability of sera from MAG vaccinated monkeys to bind mucin derived structures. Postimmune sera from all of the macaques and green monkeys vaccinated with MAG:Tn3-TT together with alum plus CpG ODN were able to recognize aOSM (Fig. 3*F*). Sera from control animals did not react with aOSM. To ensure that antibodies induced in primates will recognize human mucins, we designed several glycopeptides from human MUC1 mucin repeats. As shown in Table 2, three different MUC1 mucin sequences *O*-glyco-

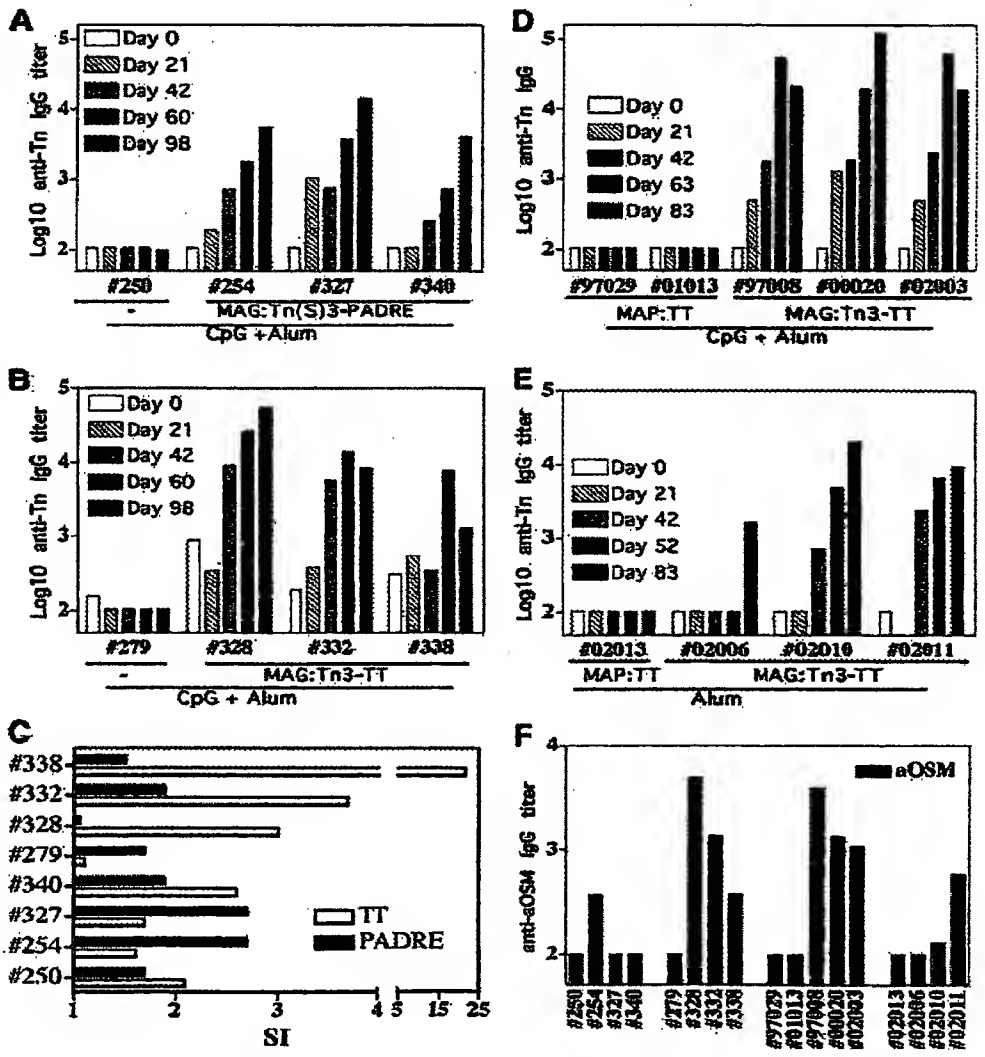


Fig. 3. Immunogenicity of multiple antigenic glycopeptide (*MAG*) in different nonhuman primate species. *A–C*, on days 0, 21, and 42, macaques were immunized with MAG:Tn(S)3-PADRE or MAG: Tn3-TT mixed with alum and CpG-ODN as adjuvant or with adjuvant alone. *D* and *E*, on days 0, 21, and 42, green monkeys were immunized with MAG:Tn3-TT or nonglycosylated MAP:TT control mixed with alum together with (*D*) or without (*E*) CpG-ODN as indicated. *A*, *B*, *D*, and *E*, at the indicated time points, sera were collected and tested for anti-Tn reactivity against the synthetic homologue, Tn3 or Tn(S)3. *C*, peripheral blood lymphocyte from macaques collected at day 60 were stimulated for 4 days with the TT or the PADRE peptide, and cell proliferation was measured by [$^3$H]thymidine incorporation over the last 16-h. For each monkey, results are expressed as stimulation index (*SI*) corresponding to the number of cpm obtained with cells incubated with the peptide divided by the number of cpm obtained with cells incubated in medium alone. *F*, sera from macaques or green monkeys collected just after the last immunization were tested for anti-aOSM reactivity by ELISA.

Table 2 *Analysis of the recognition of glycosylated mucin-derived peptides by sera from green monkeys*

| Competitor[a] | Green monkey[b] | | | | | |
|---|---|---|---|---|---|---|
| | #97008 | #00020 | #02003 | #02006 | #02010 | #02011 |
| S*T*T* G6KG | 1 | 1 | 1 | 1 | 1 | 1 |
| STAPPAHGV | - | - | - | - | - | - |
| S*T*APPAHGV | 1.3 | 0.8 | 2.2 | 0.6 | 0.9 | 1.5 |
| ALGSTTPPA | - | - | - | - | - | - |
| ALGS*TT*PPA | 86.6 | 275.1 | 45.5 | 51.6 | 16.6 | 70 |
| ALGS*T*TPPA | 169 | 446.5 | 37.8 | 37.3 | 15.9 | 46.1 |
| ALGS*T*T*PPA | 77.5 | 458.1 | 34.1 | 29.9 | 12.9 | 31.6 |
| SLSYTNPAV | - | - | - | - | - | - |
| SLS*YT*NPAV | 96.8 | 233.6 | 37.5 | 80.8 | 30.2 | 102.7 |

[a] Peptides or glycopeptides were tested in an inhibition assay to compete with a reference glycopeptide (S*T*T*G6KG) for the binding to each serum (see "Materials and Methods"). The $IC_{50}$ value was determined for each competitor and normalized with the $IC_{50}$ obtained for the reference glycopeptide to compete with itself. Results are expressed as the mean of two to three experiments.

[b] Sera from green monkeys (see legend Fig. 3) were tested for the recognition of glycosylated or nonglycosylated human MUC1 mucin peptides (* indicates α-DGalNAc).

[c] Because no inhibition was obtained with nonglycosylated peptides, $IC_{50}$ and ratio could not be calculated (- indicates the lack of inhibition).

sylated with GalNAc residues were positively recognized by sera from green monkeys immunized with MAG:Tn3-TT. Sera recognized Tn when located in the middle of the MUC1 peptide backbone, although less efficiently than the Tn located at the edge of the peptidic chain, showing the capability of MAG-induced antibodies to recognize carcinoma-associated forms of human mucins.

**Recognition and Killing of Human Tumor Cells by Antibodies from Vaccinated Monkey.** To evaluate the antitumor therapeutic potential of antibodies elicited in primates, we assessed their ability to specifically recognize human tumor cells expressing Tn by flow cytometry. When preimmune sera from green monkeys were analyzed for Tn-positive tumor cell recognition, almost no background reactivity was found for the Jurkat T lymphoma (Fig. 4, *A* and *B*), and a low reactivity was found for the MCF7 mammary adenocarcinoma (Fig. 4*C*). Likewise, postimmunization sera from MAP:TT vaccinated controls did not show any increase of reactivity as compared with preimmune sera. In contrast, after MAG:Tn3-TT vaccination, a strong and specific recognition of Jurkat cells was found (Fig. 4, *A* and *B*). MCF7 was also specifically recognized by these postimmune sera (Fig. 4*C*) but to a lesser extent as compared with Jurkat, which may reflect the level of Tn expression on these two cell types. Positive reactivity of immune sera was also found with other human Tn-expressing tumor cell lines, the T47D breast carcinoma and the LS180 and LSC colon carcinomas (Fig. 4*D*). Tn-negative T2 thymoma and MDA231 adenocarcinoma did not show any difference of reactivity between pre- and postimmune sera fully confirming the Tn-specificity of antibodies elicited after MAG:Tn3-TT vaccination (Fig. 4*D*). These results show the high specificity of anti-Tn antibodies induced by the MAG:Tn3-TT and their capacity to recognize the native Tn antigen on tumor cells.

We then analyzed the ability of sera from green monkeys to mediate killing of Tn-positive human tumor cells performed by human NK cells. As shown in Fig. 5, NK cells showed cytotoxicity against Jurkat cells coated with postimmune, but not with preimmune, sera from 5 of 6 animals vaccinated with MAG:Tn3-TT. In contrast, pre- and postimmune sera from the 3 animals immunized with MAP:TT devoid of Tn antigen did not mediate any specific killing of Jurkat cells. Because a direct killing of MCF7 cells by NK

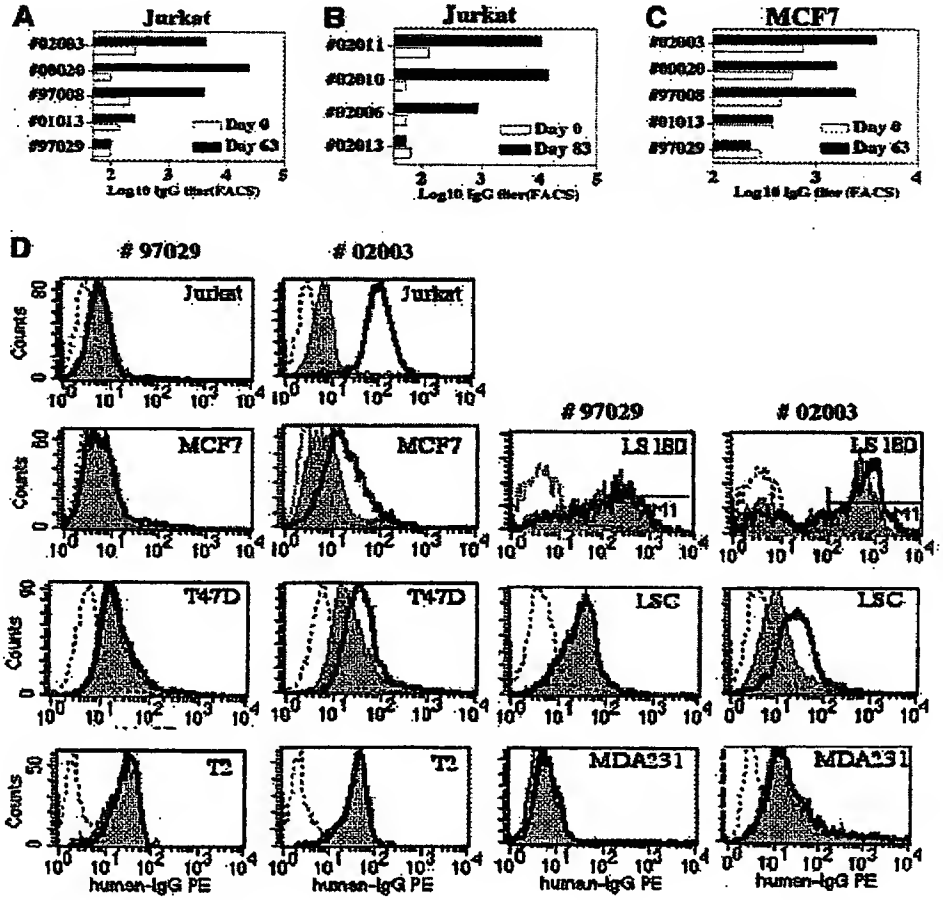


Fig. 4. Multiple antigenic glycopeptide-induced primate antibodies recognize human tumor cells expressing Tn. *A–C*, preimmune and postimmune sera obtained from the indicated green monkeys after immunization protocol detailed in Fig. 3 were analyzed for antibody titer against Tn-positive Jurkat (*A* and *B*) and MCF7 (*C*) cells. *D*, fluorescence-activated cell sorting (*FACS*) histograms for cells stained with secondary reagent alone (----), with preimmune sera (*gray histograms*), or postimmunization sera (*bold line*) collected from indicated green monkeys and tested against Tn-positive [Jurkat T lymphoma; MCF7 and T47D mammary adenocarcinomas; LS180 and LSC colon carcinomas or Tn-negative tumor cell lines (T2 and MDA231)]. For LS180, the percentage of positive cells (gated in M1) observed for pre and postimmune sera is 50% and 52%, respectively, for #97029 and 55% and 75%, respectively, for #02003.

cells was observed in the absence of any antibody (data not shown), it was not possible to assess ADCC against this tumor cell line. Altogether, these results show that the MAG is capable to induce antibodies specific for Tn on human tumor cells and to mediate killing of these cells via an ADCC mechanism.

## DISCUSSION

In the present study, we elaborated a candidate vaccine targeting the Tn tumor-associated carbohydrate tumor antigen for immunotherapy of carcinoma cancer. We designed a suitable glycotope cluster containing three α-GalNAc on a STT backbone, and we show that the MAG has superior immunogenic potential over a KLH-Tn glycoconjugate. We also show that the MAG designed for human therapy is able to induce anti-Tn IgG antibodies in two nonhuman primate species that can mediate ADCC against human tumor cells.

The Tn antigen displayed on carcinoma-associated human mucins is preferentially displayed as a cluster of several Tn (16–18), and this configuration is optimal for recognition of Tn by IgG antibodies to mediate ADCC against cancer cells. We showed previously that a cluster of three Tn is very efficient in stimulating anti-Tn antibodies capable of recognizing native tumor forms of Tn and eradicating Tn-expressing tumors in mice (23). The cluster we introduced in the MAG is a tri-Tn cluster (three α-GalNAc on a STT backbone) corresponding to a glycotope recognized by the MLS128 monoclonal antibody that has been obtained after immunizing mice with the human carcinoma cell line LS180 (39, 40). Amino acids flanking the Tn antigen have been shown to modulate antibody recognition (41, 42), but nothing is known about the contribution of the aglyconic part of the Tn structure (Ser or Thr residues) for antibody binding. To design the most suitable vaccine candidate for targeting immune responses to cancer cells, we first investigated the influence of the amino acid backbone displaying the GalNAc residue. We show for the first time that antibody recognition of the α-GalNAc moiety of Tn is influenced by the aglyconic part of the Tn structure. Indeed, Tn displayed on a non-natural homoserine residue was not the most appropriate to induce anti-Tn antibodies that recognize the native form of Tn. Likewise, antibodies induced by Tn clusters based on natural amino acids (Ser or Thr) failed to efficiently recognize the GalNAc residue display on a homoserine backbone. The influence of the amino acid backbone was less sensitive when *O*-GalNAc residues were displayed by natural amino acids for polyclonal antibodies. However, among Tn-specific monoclonal antibodies we produced, we found some exclusive fine specificity for Ser or Thr. It remains that the diversity of the polyclonal response allows a clear cross-reactivity between anti-Tn antibodies raised by *O*-GalNAc residues carried by a SSS, a STT, or a TTT backbone for heterologous backbones. The STT backbone was found to be the most permissive for induction of anti-Tn antibodies that recognize degenerated Tn clusters. These anti-Tn antibodies recognize a large variety of GalNAc glycosylated MUC-1 peptide sequences allowing a broad spectrum of recognition for native forms of Tn on cancer cells. Antibodies induced by a Tn3 cluster on a STT backbone can efficiently recognize a large variety of MUC-1 peptide sequences with different levels of GalNAc glycosylation. Given the large heterogeneity of mucin sequences, it is critical to build immunogens capable to induce anti-Tn antibodies with a broad spectrum of recognition for native forms of Tn.

The second important point highlighted by the present study is that the full synthetic MAG:Tn immunogen is able to induce anti-Tn IgG antibodies with a mild adjuvant setting (alum), whereas the KLH-Tn conjugate requires the use of a much powerful adjuvantation, such as QS21. Likewise, glycolipopeptides based on dimeric or trimeric Tn cluster induced IgM, but no or low IgG anti-Tn antibodies (21, 29). In contrast,

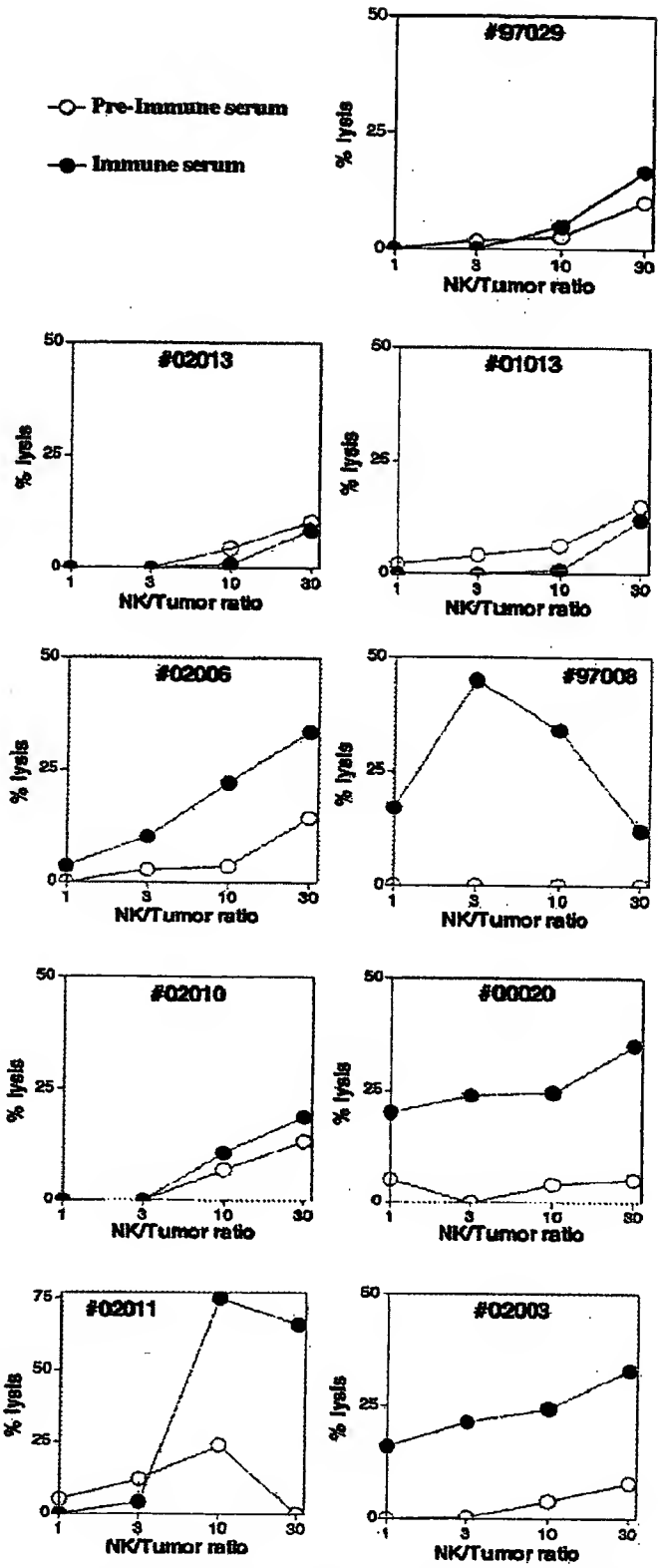


Fig. 5. Multiple antigenic glycopeptide-induced primate antibodies are able to mediate antibody-dependent cellular cytotoxicity against Tn-positive tumor cells. [$^{51}$Cr]-labeled Jurkat cells were incubated with pre- or postimmune sera from green monkeys injected with MAG:Tn3-TT or control MAP:TT as detailed in Fig. 3. These target cells were subjected to lysis by the natural killer cell clone at the indicated natural killer (*NK*):tumor ratio for 4 h.

the MAG:Tn3-PV mixed with a lipopeptide adjuvant induced strong anti-Tn IgG antibodies (data not shown). Therefore, the MAG system offers the possibility to induce a strong antitumoral immune response with a well-known, commonly used and harmless adjuvant.

The last important point provided by the present study is that two different MAG designed for human therapy by introducing promiscuous T-helper HLA-DR binding peptides are able to induce anti-Tn antibodies in two nonhuman primate species. The two different CD4 T-cell peptides, PADRE and TT, introduced into the MAG were capable to provide help for anti-Tn antibody production in all of the immunized primates. Again, these antibodies could be induced with a mild adjuvant setting (alum), although the addition of CpG oligonucleotides strongly improved the immunogenicity of the MAG by eliciting a quantitatively higher and more rapid response. Importantly, in all of the experimental settings, no adverse reaction was observed in any animal such as local inflammation at the sites of injection or weight loss assessing the safety of the MAG. Tn-specific antibodies elicited in these nonhuman primates were able to recognize glycosylated human mucin sequences as well as Tn-positive human tumor cells. Antibodies specific for tumor-associated antigens are able to mediate tumor cell killing by complement-dependent cytotoxicity or by ADCC. Importantly, these antibodies in the presence of human NK cells could mediate ADCC against tumor cells demonstrating their antitumoral potency. In conclusion, we have designed and validated a fully synthetic vaccine targeting the carbohydrate Tn tumor antigen for immunotherapeutic purposes in humans, opening the way for a new generation of vaccines based on fully synthetic glycopeptides.

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